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Reprocessing: right to pause and think

HISTORY will almost surely record that the 1970s were years when the industrialised world had more than one occasion to pause and think about where it was going, and where it was taking everyone else. With the over-riding disposition still to 'go for growth' and environmental issues and energy problems assuming greater importance, it doesn't take a wise man to spot that just such an opportunity is now with us. Fortunately it looks as though the west's leading powers think so too.

The issue that prompts a pause is the reprocessing of nuclear fuel. As the following articles indicate, the question of whether to push ahead with this activity still further is now under scrutiny in Britain and the United States. A US moratorium on reprocessing and the export of reprocessing technology, called for by President Ford in October, has been followed by a similar decision from France last week. And an announcement is expected from Canada before the end of the year.

As with all international issues, these developments have aroused some suspicion about the motives behind them; not every country, after all, is at this moment equally well placed to reprocess nuclear fuel. But if not too much ought to be made of such events, it is nevertheless right that they have occurred. The three contemporary concerns of growth, energy and the environment are now acting to reinforce each other in the public arena, and the focus they have found is nuclear power.

At the heart of it all is the question of whether a fast breeder reactor system should be developed, but that question, though important, is receiving somewhat disproportionate attention. This may be because of a misguided belief that a decision on the breeder is a decision on the future of reprocessing. If the breeder doesn't receive the green light it does not automatically follow that reprocessing fuel from thermal reactors as a

commercial operation has no purpose. What is often forgotten is that plutonium, the retrieval of which is the principal object of reprocessing (though obviously the uranium obtained is useful too), can also be used in oxide-fuelled thermal reactors. That means a decision on reprocessing, the most radioactive stage in the nuclear fuel cycle, is quite as important as a decision on the breeder.

To see better why this is so needs some elaboration. Reactors in service now use either natural uranium metal fuel (Magnox), natural uranium oxide (CANDU) or slightly enriched uranium oxide (AGR and PWR). Spent fuel taken from Magnox reactors and put to 'cool' under water can, after a period that ought not to exceed about a year because of corrosion, either be reprocessed to yield practically all the uranium and plutonium, together with high level waste which remains active for hundreds of years, or dealt with directly as high level waste which remains active for thousands of years.

The waste after reprocessing is at the moment kept in enormous stainless steel tanks. Techniques to render it safer and easier to handle through a process of glassification are under development, and reliable sites for its disposal are also being sought. The problems are essentially the same whether reprocessing is done or not. The increase in volume of waste that has to be handled is obviously considerable if reprocessing is not done, and the much longer period for which unprocessed fuel remains active means that prospective sites must be correspondingly more reliable geologically: but these differences are almost marginal at this stage, and offer only a rather perverse case in favour of reprocessing.

In practice there is little realistic, or economic, option but to reprocess the Magnox fuel. The engineering problems associated with the vitrification technique have yet to be ironed out, and as the spent fuel elements build up in the

cooling ponds there is a greater danger in not reprocessing as long as alternative ways of keeping the spent fuel are unavailable. Both Britain and France are reprocessing Magnox fuel on a commercial scale to retrieve uranium and plutonium. To that extent they are already in the so-called 'plutonium economy', which so many people wrongly believe is something which only comes in with the fast breeder.

The spent fuel of both CANDU reactors and of AGRs and PWRs, like that of Magnox-fuelled reactors, contains plutonium and uranium; uranium from AGR and PWR fuel is richer in uranium-235 than natural uranium, and this gives it extra value. What makes the question of oxide fuel reprocessing different from any question associated with Magnox fuel reprocessing is that the need to reprocess oxide fuel is not urgent. Indeed, with the right precautions, spent oxide fuel can be stored under water for a matter of years if not decades without significant danger, although those arrangements obviously should not be permanent and would create surveillance problems.

No commercial-scale oxide reprocessing plant is yet in operation, and on present calculations none is expected to begin before the 1980s. But the case for having one is now being pressed heavily by the nuclear industry. At one level the arguments make the point that the hazards attached to the process are not greater than with the already-accepted Magnox fuel reprocessing, save that oxide fuel is substantially more radioactive and produces more waste afterwards. Important as that is, as a matter of industrial danger the point is separate from the worries about plutonium, which are not answered simply by the fact of what is, after all, an imposed choice regarding the reprocessing of Magnox fuel.

The case for oxide fuel reprocessing is anyway being made at another level. It turns on the already-mentioned fact that plutonium does not just have military purposes when it is not used to



fuel breeder reactors. Oxide-fuelled thermal reactors can be adapted to take a mixed oxide (plutonium oxide-uranium oxide) fuel. Whereas the breeder uses 20% plutonium oxide, though, the adapted thermal reactors would use something closer to 2-3% plutonium oxide.

The plutonium would be used less efficiently than in a breeder, but this 'plutonium recycle', together with enrichment of the retrieved uranium (an operation presenting no new hazards), would clearly extend the life of available nuclear fuel. To reprocess, in other words, is to lengthen the period for which the world has nuclear power, quite regardless of the fast breeder, at a time of burgeoning uranium prices and limited uranium supplies.

It is a nice argument, because it emphasises both economy and resource use. But the additional period for which the world would have nuclear power is disputed. It is certainly not long by comparison with the supposedly limitless extension that a fast breeder system would allow. Indeed, the uranium saved may not be at all great given the massive fuel-consuming reactor programmes envisaged for the future; and the money saved will, in many people's estimates, be quite marginal by comparison with the huge capital costs involved in generating nuclear power—costs which won't decline while engineering problems, licensing requirements and environmental regulations grow.

If the broad economic case for reprocessing looks unproven that may be because too much depends on what price people are willing to pay. But that in turn means assessing what risks people are prepared to take. The same is true of the arguments about resource use, which raise the very questions of moral responsibility that environmentalists who campaign against oxide fuel reprocessing would urge be taken into account. The industry says it is irresponsible not to prepare for the fast breeder option if and when it comes, and not to use plutonium as a fuel in thermal reactors if not breeders. The response it receives is that people must first decide, on the basis of an assessment of risks involved.

There is evidence that this is now happening. Certainly many of the worries about plutonium have been well publicised. The industry acknowledges the problems but says they are not insuperable and ought not to worry people as much as they do. There is indeed a need for greater perspective—the risks associated with overeating, drinking, smoking and driving, and from existing toxic substances, are probably greater. But when it comes to proliferation, it is another matter.

The industry says the safest place for retrieved plutonium is in a reactor, and sees the move to oxide reprocessing as a change of degree rather than kind. But it is precisely the change in scale that is worrying many people, since there is hardly likely to be a greater insurance against danger with the wider use of larger amounts of plutonium. The serious aspect of reprocessing, though, concerns the international trade in the technology, for this is the nub of the proliferation problem. The capacity of any country to produce nuclear weapons is eased considerably if it has its own reprocessing plant.

Among the countries now operating or building experimental or pilot-scale plants are West Germany, Japan, India, Italy, Spain, Yugoslavia, Argentina and Taiwan. Brazil is expecting a plant from Germany, and Pakistan is expecting one from France. Whatever the fears about the use by terrorists of plutonium, the fact remains that it is governments which have made and used bombs in the past, and governments which are likely to make and use them in future. Governments are sovereign to sufficient degree that even the best safeguards can be flouted that depend finally on faith for their effectiveness. Today's government may in any case be tomorrow's guerrillas.

The discussions of the Nuclear Suppliers Group—the exporters of nuclear technology—are thus too important to take place in the secrecy that has beclouded them so far. The lack of information on these talks has helped to prevent the discussion of the issues which is now necessary. But even if the group manages to agree to abandon exports of reprocessing technology, it must acknowledge that other countries have nuclear programmes, and will then have to decide whether reprocessing, if it is to be done at all, is to be done at an international reprocessing centre.

The industry's argument, that proliferation is here to stay anyway, is strong. There is, it says, enough published on the subject of reprocessing for a determined nation with a nuclear reactor to have a small-scale plant secretly (if inefficiently) helping to make weapons, at not too prohibitive a cost. There are also probably enough people in the business who know about the subject to assist in such a cause. And who would dare stand up and say that other countries should not have nuclear power in the first place?

If this is unanswerable, other questions do need a proper response before anyone concludes that an international reprocessing centre is the best solution. Will the creation of such a centre remove the incentive for others to re-

process altogether, or because of its attractions (and the precedent) encourage others to do it too? And will it induce other countries justly seeking energy independence to go nuclear over-hastily, or deter them completely?

And the prior question of whether there should be reprocessing at all must be answered first. A decision against reprocessing, if sustained, would be a decision against a nuclear future, and to that extent against nuclear power. But at least it would be a reversible decision which would not later preclude lengthening the resulting nuclear interlude finitely (by allowing reprocessing to go ahead) or almost infinitely (by allowing a system of fast breeders). A decision the other way permits no such flexibility.

Given the world's energy problems, though, there is a clear need for early decisions to be made at the highest levels on the subject of reprocessing. The powerful multinational nuclear industry, of course, though it has grown encouragingly more sensitive to public opinion, wants quick decisions because of the long lead-times on its investments and because it wants to secure the exports it needs to survive. But these should still only be made after the fullest possible informed debate before the widest possible public, a requirement that has not yet been fulfilled. This is the increasingly salient 'legitimacy factor' in the nuclear business; it demands that consent be mobilised through the usual channels of public debate, be they letters to local papers or national referenda.

A decision not to reprocess at all would imply a particular view about world energy which the industry cannot accept. For the vast majority of the world's population, that view may not be as gloomy as it has been painted. Their needs, it is often said, would be served by eschewing nuclear energy in favour of natural sources appropriate to a course of economic development that does not duplicate the course already taken by the industrialised world.

Perhaps in this perspective, which comes from those concerned at the widening gap between developed and underdeveloped nations, there is something for the industrialised world to learn. After all, if the energy crisis is as serious as people say, it ought to be tackled seriously, and not simply with proposals that unquestioningly offer more of the same. The reprocessing of oxide fuel is an issue which provides a suitable occasion for some of these matters to be raised. It may also be the single most important nuclear issue facing the world at this moment which is also amenable to political influence. The question is, is reprocessing worthwhile? It is at least right to pause and think. □

Reprocessing (1): Barnwell, USA

An inevitability forestalled

Colin Norman, recently at Barnwell, reports
on the reprocessing debate in the United States

AMONG the more controversial problems awaiting the attention of the Carter Administration next year will be whether to allow an extraordinary nuclear facility, built like a concrete fortress in the middle of a South Carolina pine forest, to start operating. Constructed by private industry at a cost so far of some \$250 million, the facility could become either a key component in the nuclear power programme in the United States, or a massively expensive white elephant.

Designed as the first full-scale commercial plant to reprocess spent nuclear fuel from power reactors, the facility is already falling victim to rising concerns about the proliferation of nuclear weapons, and its fate will probably hang on a complex series of regulatory, political and diplomatic decisions expected to be taken next year. But, even if the Carter Administration does decide to allow the plant to begin work, its troubles won't be entirely over. Before it can operate at full capacity, additional facilities to solidify highly radioactive wastes from the plant and to convert plutonium nitrate to plutonium oxide will be required. They could cost as much as \$500 million, a sum which private industry says it is unwilling to risk without government assistance.

When plans for the plant were drawn up in the 1960s, it looked like a sure-fire commercial proposition. Opposition to nuclear power had scarcely begun to surface, prospects for burgeoning growth in the nuclear industry looked good and reprocessing was generally regarded as inevitable. Three industrial concerns—the Allied Chemical Company (50%), Gulf Oil (25%) and Royal Dutch Shell (25%)—therefore decided to form a partnership, called Allied General Nuclear Services (AGNS), and sink about \$100 million into the venture. They expected to have the facility in operation by about 1974.

Enticing idea

The plant is designed to reprocess fuel continuously from about 50 commercial power reactors, extracting plutonium and uranium from highly radioactive spent fuel rods and recycling the materials as new reactor fuel. The idea looked enticing.

Light water reactors now in use in the United States and in most other

countries are fuelled with uranium in which the concentration of the fissile isotope uranium-235 has been enriched from its natural level of about 0.7% to about 3%. While the reactor is operating, small quantities of fissile plutonium are formed along with other highly radioactive products and when the fuel rods are removed from the reactor, they contain roughly 1% plutonium and 1% unused uranium-235—more than half the original amount of fissile material. If those elements could be extracted and recycled as reactor fuel, savings in fuel costs could be substantial, and the need to mine and enrich uranium would be greatly reduced.

Another incentive for commercial concerns to get into the reprocessing business was the expectation that large numbers of breeder reactors would be in operation by the end of the century. Since breeder reactors produce large quantities of plutonium, a plutonium extraction industry is essential to the economics of their operation. Small wonder, therefore, that Allied, Gulf and Shell decided to get into the reprocessing business.

They were not alone when they designed the plant in the 1960s. A small-scale reprocessing facility was already in operation at West Valley, New York, and another was on the drawing board for a site at Morris, Illinois. In addition, plutonium had been extracted from spent fuel from military reactors ever since the Manhattan Project in the early 1940s—a fact that gave would-be commercial reprocessors confidence that the process would work successfully. So far, however, the nascent reprocessing industry has been beset by disasters.

The West Valley plant, designed to handle about one ton of irradiated fuel per day, was operated from 1966 to 1972, when it was closed for modification and expansion. Since then, the estimated costs of modifying the plant have skyrocketed, uncertainties have arisen about how the wastes should be handled, and the amount of time required to complete the construction work has stretched to ten years. Consequently, the plant's operator, a consortium known as Nuclear Fuel Services, announced last year that the plant would be scrapped.

The second facility, constructed in the early 1970s by the General Electric Company at Morris, was an even

greater disaster. General Electric sank about \$64 million into the plant, but a number of problems with remote handling of radioactive material were encountered during testing and in 1974, the company decided to cut its losses and abandon the venture.

The AGNS facility is therefore the only plant remotely close to operating, though the Exxon Nuclear Company has applied for a licence to construct a similar-sized plant at Oak Ridge, Tennessee, in the mid-1980s. The facility, located near Barnwell, South Carolina, is generally acknowledged to be vastly superior technically to the West Valley and Morris plants; it is, however, facing a determined challenge from nuclear critics and arms control advocates, who argue that it would usher in a new and more dangerous nuclear age—the so-called plutonium economy. Opposition to the facility has, in fact, already caused long delays and is partly responsible for the cost escalation from the original estimate of \$100 million to the present cost of \$250 million.

Massive construction

Built to reprocess about 1,500 tons of irradiated fuel per year—the output from some 50 reactors—the facility is certainly impressive. A massive concrete construction, lined throughout with stainless steel, it is designed to operate by remote control. Irradiated fuel rods will be shipped to the plant in heavily shielded casks, and they will be stored in the facility in huge water pools. From there, they will be transferred to a giant hydraulic shearing device, which will chop the rods into two-inch sections, and the pieces will be plunged into a bath of hot, concentrated nitric acid.

The acid bath will dissolve uranium, plutonium and other radioactive elements from the fuel rods, leaving behind the stainless steel cladding, which will be removed and stored pending final burial. Plutonium and uranium will be removed from the nitric acid solution by a relatively straightforward solvent extraction process, and the plutonium and uranium salts will be separated from each other electrolytically. The waste solution will be concentrated and transferred to huge, double-walled stainless steel tanks for temporary storage.

A facility for converting uranyl nitrate from the separation plant to uranium hexafluoride has been built by AGNS at the Barnwell site, the idea being to ship the fluoride directly to an enrichment plant at Oak Ridge, Tennessee. As for the plutonium, the plan is to convert it eventually to plutonium oxide for fabrication into reactor fuel consisting of a mixture of plutonium oxide and enriched uranium

oxide. At present, however, no plutonium conversion facility has been built at Barnwell, and the element will therefore probably be stored temporarily as plutonium nitrate solution in titanium tanks.

Plutonium politics

A number of environmental and anti-nuclear groups, led by the Natural Resources Defense Council (NRDC), began to worry in the early 1970s about the consequences of pushing ahead with a programme to extract and recycle plutonium on a large scale. They argued that plutonium could be stolen from the nuclear industry by individuals or terrorist groups and fashioned into crude atomic weapons, and they also challenged the federal government's exposure standards for plutonium, arguing that they are much too lax. So far, they have achieved considerable success in their assaults on the programme.

In 1974, the Atomic Energy Commission, which was then responsible for licensing nuclear facilities, announced that it would publish an environmental impact statement on the potential consequences of a large scale plutonium recycling industry before deciding whether to give the Barnwell plant a licence to begin operating. The statement was published in August, 1974, and it recommended giving a green light to plutonium recycle.

A few months later, however, the Atomic Energy Commission was scrapped and its licensing functions were transferred to the Nuclear Regulatory Commission (NRC). The new regulatory environment hasn't helped the Barnwell plant. In January last year, the NRC announced that it would re-examine the AEC's impact statement, and publish an expanded version of its own. NRC also announced that it would subject the matter to an exhaustive public hearing before making any decision on reprocessing and recycling.

The first part of NRC's impact statement, dealing with health and safety questions, was published a few weeks ago, and the second part, covering the more sensitive issues of safeguards and cost-benefit analysis, is expected to be published early next year. The promised public hearings have begun, and they are expected to continue for most of next year. NRC officials say that they hope to reach a final decision on whether reprocessing and recycle should be allowed by January 1978, which means that even if Barnwell gets a green light, it would not be started up until 1979.

In the meantime, however, the regulatory process has been overtaken to some extent by political developments. The explosion by India of an atomic

device constructed from plutonium extracted from an imported nuclear reactor in 1974 set loose a major debate in the United States about the nation's responsibilities to try to curb the spread of nuclear weapons. Throughout the past year, US officials have been urging the adoption of strict international controls on nuclear exports and they have been arguing for a complete moratorium on the sale of reprocessing and uranium enrichment technology to nations which do not have nuclear weapons. Concerns about proliferation have, in turn, led to a re-examination of the United States' own plans for reprocessing, and the matter even played a prominent role in the Presidential election.

In a speech delivered at a United Nations meeting last May, President-elect Jimmy Carter announced that he had reservations about pressing ahead with plutonium recycle in the United States while the State Department is trying to persuade other nations not to follow the same path. In November, Carter was more specific. He said he would "seek to withhold authority for domestic commercial reprocessing until the need for, the economics, and the safety of this technology is clearly demonstrated. If we should ever decide to go forward with commercial reprocessing, it should be on a multinational basis". A few days before the election, President Ford came out with a similar statement. The nuclear industry in the United States should no longer assume that reprocessing will be "a necessary and inevitable step in the nuclear fuel cycle", he said, and he announced that he would permit reprocessing and plutonium recycle "only if they are found to be consistent with our international objectives".

Mixed reaction

Those statements were greeted with satisfaction by opponents of the Barnwell plant. Nuclear critics and arms control advocates had long been arguing that a decision by the United States to forego reprocessing, at least for the time being, would send a clear message abroad that the United States is serious about its non-proliferation objectives. Needless to say, the nuclear industry doesn't agree.

Dr J. A. Buckham, manager of the Barnwell plant, said in an interview last week that "almost all of the stated objections of opponents of reprocessing are best served by the very opposite of what they advocate". He argued that the best way to persuade non-nuclear countries against embarking on reprocessing is to ensure that reprocessing services are available for all, perhaps on a multinational basis. "The failure to start up this plant (Barnwell) will not persuade the rest of the world not

to reprocess", he said, and added that the plant is so well safeguarded that it would not add to the proliferation problem at all.

Buckham also noted that reprocessing and plutonium cycle would stretch out uranium reserves and to help reduce reliance on fossil fuels. In other words, it is a key to the long-term prospects of the nuclear industry, which is one reason why nuclear critics are so opposed to the operation.

It should be noted that neither Ford nor Carter entirely ruled out the possibility of operating the Barnwell plant. Carter suggested that it could be turned into a multinational reprocessing facility, and both argued that the economics and safety of reprocessing should be proven before embarking on a full-scale reprocessing industry. The nuclear industry argues that the best way to prove the technology would be to operate the Barnwell plant as a demonstration facility, with government assistance. That possibility was under intense consideration in the Ford Administration last year, and it hasn't been entirely ruled out.

Operation of the plant would, however, require considerable capital expenditure. At present, it has capacity to store plutonium nitrate from about 18 months' production. A plant to convert the material to plutonium oxide would therefore be required soon after the facility is put into operation. Similarly, there is only temporary storage capacity for about one year's worth of radioactive wastes, and a solidification plant is required. Those plants could cost as much as \$500 million.

In view of the uncertainties surrounding the future for reprocessing, AGNS is unwilling to make that large an investment, and it has asked the Energy Research and Development Administration (ERDA) to build the ancillary facilities. The company said that it would be prepared to buy the facilities from the federal government once they were licensed and the plant was in full operation. ERDA officials have indicated that though federal assistance would not be forthcoming immediately, the possibility has not been ruled out entirely.

The future for reprocessing in the United States is therefore uncertain. Nuclear critics, such as Arthur Tamplin of NRDC, are convinced that the Barnwell plant can be stopped, while AGNS officials predict that they will receive a favourable decision from NRC. The matter is likely to be decided eventually by Congress—particularly if federal assistance is required—and it should be noted that the climate for nuclear power there is likely to change next year with the pending demise of the Joint Committee on Atomic Energy. □

Reprocessing (2): Windscale, UK

'The β -in-air alarm is a yodel sound'

Gillian Boucher, recently at Windscale, reports on the reprocessing issue in Britain

PRESS reaction to the leak at the Windscale reprocessing plant in Cumbria have confirmed British Nuclear Fuels (BNFL) in its belief that the media are impossible. The recently reported leak of 100 gallons a day of weakly radioactive water from an old concrete silo containing solid wastes stored in water was discovered on October 10 and its source has still not been identified. But in spite of shrieks about contamination it seemed clear almost from the start that there was probably no danger to the health and safety of workers or anyone else.

There have been allegations of secrecy but the UK Health and Safety Executive is satisfied that BNFL carried out its only obligations in informing the Nuclear Installations Inspectorate after a few days (as the leak was not dangerous there was no obligation to report it immediately) and the unions at the beginning of November. Mr Anthony Wedgwood Benn, Secretary of State for Energy, to whom BNFL is responsible, learned of the leak on December 8 and informed the House of Commons the next day. He later gave the impression that BNFL had improperly withheld information, and is now insisting on receiving details of all future incidents as soon as they occur. But in fact it was the Health and Safety Executive, not BNFL, that was responsible for reporting accidents to Mr Benn, and its rule of thumb has hitherto been that if they are not dangerous he need not be told. BNFL may, therefore, have suffered more than it deserves.

The painful lesson BNFL may now be learning, however, is that it is bound to be accused of secrecy unless it willingly provides far more information than it is statutorily obliged to do. Unless it leaves as wide a margin in its obligation to inform as it does in its compliance with safety regulations the criticisms will continue unabated. In this instance BNFL would have spared itself some insults and probably not lost anything if it had informed Cumbria County Council of the leak, which was discovered while the council was considering BNFL's application for planning permission for a major expansion programme. BNFL's rather lame excuse was that the leak was such a clear indication of the need for new plant

that revealing it could be seen as twisting the council's arm.

Three decisions

Mr Benn's strong reaction is perhaps best explained by the three major nuclear decisions the government will soon have to make, or at least postpone, in the face of rapidly growing public concern. The first is whether to allow the expansion at Windscale that the Cumbria council has provisionally approved: refurbishing the Magnox fuel reprocessing plant, building a new oxide reprocessing plant and assorted research work including development of the vitrification of wastes. Mr Shore, Secretary of State for the Environment, may announce a decision on this this week. The second is whether to build the first commercial fast breeder reactor. Mr Benn is now saying that there is plenty of time to consider this and a decision may not be forthcoming for a year or two. The third is whether to continue with the commitment to use the British steam-generating heavy water reactor (SGHWR) for the next generation of nuclear reactors. At present Mr Benn seems likely to opt for another large coal-fired power station whose cost (£600 million) will preclude development of the SGHWR programme. The SGHWR programme is also threatened by the latest round of public spending cuts which will probably mean delays in nuclear development, and by the Central Policy Review Staff's analysis of the power plant manufacturing industry, released last week, which points out the poor export prospects for the SGHWR.

Mr Shore, having postponed a decision on the Windscale expansion, now has several choices. He can refer the matter back to the county council, call in the matter for his own decision—either positive or negative—or set up one of two types of planning inquiry. The first would consider only third parties affected by the proposals, that is, local people; the second, a Planning Inquiry Commission, would look beyond the purely local issues.

Strong pressure to say yes immediately has come from BNFL management and unions because of £600 million-worth of deals in the pipeline to reprocess oxide fuels from abroad which could be jeopardised by delays. According to

Con Allday, BNFL's Managing Director, prolonged delay would mean disaster. Mr Shore is under pressure from other departments including the Treasury and the Department of Industry not to forego the foreign earnings. Mr Benn in effect gave the green light last March when he permitted BNFL to seek overseas business and to continue negotiations with the Japanese over the reprocessing of their oxide fuel. The government has not authorised the use of resources in this connection, and Mr Benn's recent enthusiasm for answering parliamentary questions embarrassing to BNFL suggests that he has misgivings. Mr Shore's inclination may yet be towards an inquiry, if only because it will be very difficult for him to call in any other planning application if he lets this one go. And the public hostility to nuclear power that has been fanned by the news of the leak is bound to influence his thinking.

Growing controversy

A year ago a decision not in BNFL's favour seemed inconceivable; nuclear power was not the issue in Britain that it was in the United States. Though the questions have remained essentially the same the number of people talking about them has greatly increased; certainly the opposition has had a better press than BNFL. And a shadow of concern on the part of such a body as the Flowers Commission perhaps changes more minds than the clamouring of half a dozen pressure groups.

BNFL insists that reprocessing is necessary for waste management, but for oxide fuels, which can be stored for decades, the requirement is not an urgent one; if the plutonium were not to be used—the controversial question—one eventual possibility might be to reduce the bulk of the spent fuel by removing the uranium and to vitrify the plutonium together with the wastes. Aside from plutonium, it is profits that make BNFL interested in reprocessing now.

The proposed expansion will cost £600 million of which half will be for the oxide plant; but a large part of the capital costs would be met by future customers for oxide reprocessing. The rest is expected to come from retained profits and loans from the private sector; £100 million was raised recently from a consortium of British and foreign banks. As the fuel from the British oxide reactor programme will only occupy 20–30% of the oxide plant's capacity there is plenty of room for those desirable export orders. An important political consideration is that expansion would provide another 1,000 jobs over the next five years to depressed Cumbria. And delays might

cause all this to be lost, possibly to the French or to the Americans, whose moratorium on foreign reprocessing extends only for three years.

But some questions remain to which it is difficult to get a clear answer. BNFL is a partner in United Reprocessors, an Anglo-French-German market-sharing body; just as the French came in on what was originally an Anglo-Japanese deal, it has not been explained what would prevent the British from demanding a share at any time in a Japanese-French deal even if there were a few months' delay for an inquiry. There would be a clause allowing the Japanese fuel to be returned un-reprocessed if the vitrification process were not developed satisfactorily, but a hefty downpayment that had meanwhile been invested in plant would be a lot to ask the British taxpayer to return to Japan. And though the job issue was until recently the one that mattered most in Cumbria, there are suggestions that the latest leak has swung many locals in favour of caution and an inquiry.

The other reason for reprocessing—the development of the fast breeder reactor which will use plutonium fuel—is itself in doubt. Even if the next stage, the CFR-1, goes through, Mr Benn's advisers are taking seriously the possibility that electricity demand will remain pretty much at present levels for the next 20 years and large capital expenditure on power stations around the year 2000 will not be necessary. There is already enough plutonium to fuel one commercial-scale fast reactor, and there are obvious disadvantages to separating out a lot more.

Safety issues

If reprocessing makes commercial sense or is necessary because of the fast breeder programme—both of which are of course hotly contested—there remain the issues of the safety of reprocessing and all that the plutonium economy entails. As far as the safety of reprocessing as an industrial process is concerned, the recent leaks look like supporting the Flowers Commission's remark that BNFL's housekeeping is not all it might be. But BNFL is careful to keep workers' radiation doses a long way within permitted limits; the yodel sound of an alarm is of course but one of an elaborate system of warnings and safeguards. So far nobody has been shown to have died as a result of his work at Windscale (the unions are at present seeking compensation for the families of three workers who have died of various cancers but BNFL is contesting the claim)—a record that few other large industries could claim. Suggestions have been published, however, that a succession of minor accidents with very similar causes shows that the

safety engineers are not learning as much from their past mistakes as BNFL claims.

Radiation levels in the Celtic Sea have caused a good deal of concern. The latest government figures show that the amount of alpha radiation is well within permitted limits but concentrations of beta emitters have risen steeply to reach a worrying 83% of permitted levels by 1975. The most dramatic increase—the tenfold rise in caesium levels in fish between 1973 and 1975—is mainly due to the corrosion of Magnox fuel elements stored for too long before reprocessing and releasing caesium into the cooling water; that should be corrected by the proposed improvements to the Magnox plant. But to increase throughput to the extent envisaged without increasing the dumping of radioactive elements in the sea will surely challenge BNFL's engineers.

Reprocessing oxide fuel is undoubtedly going to be a difficult business with very much higher levels of fission products and plutonium to deal with. Critics point to the 1973 accident at the experimental 'head-end' plant for pretreating oxide fuel which contaminated 35 men, but too much should not be deduced from what happened in a somewhat makeshift extension of the Magnox system; BNFL repeatedly point out that you cannot expect it to achieve the highest safety standards if it is not allowed to invest in new, purpose-made equipment. Many feel that if oxide fuel is to be reprocessed anywhere in the world, Windscale is probably one of the best places to do it.

Other problems, such as the storage of waste and even the protection of plutonium from terrorists, have a large technological component and could probably be solved given time and money. BNFL has suggested that plutonium fuel for fast reactors could for example be mixed with uranium oxide and poisoned with a gamma emitter to make it as unattractive a proposition for a criminal as the spent fuel which is transported unguarded from power stations to Windscale. Plants could be designed to make pure plutonium inaccessible. The unsavoury accompaniment of the plutonium economy, the big-brother surveillance, seems less of an issue in the good-humoured atmosphere of Windscale, where half the workers are locals and twenty-five years' service is not uncommon.

Critics emphasise proliferation

Friends of the Earth, the most persistent and articulate critics of reprocessing, would not accept that these problems are easily soluble, but their main point of attack is over nuclear

proliferation. BNFL complain that Friends of the Earth are always changing their ground: not long ago they attacked nuclear power plants, then they shifted to reprocessing and now they say that Magnox reprocessing is acceptable but oxide reprocessing should be prevented. Friends of the Earth reply that they learn as they go along and "try to find issues with a cutting edge within the general policy framework"—which is pro-conservation and small-scale energy production, rather than specifically anti-nuclear.

On the same side as Friends of the Earth are the myriad other pressure groups and individuals—the Conservation Society, the Lawyers' Ecology Group, Half Life, assorted academics and bishops. Ranged against them, at least in theory, is the establishment: BNFL, their owners the Atomic Energy Authority, the nuclear contractors the National Nuclear Corporation, the Central Electricity Generating Board, even some government departments. Additionally and interestingly, the trade unions have been solidly in favour of reprocessing and expansion. In practice BNFL, being the dirty end of the business, gets most of the criticism and has to put up most of the defence. In between, and supposedly helping in the nuclear debate that Mr Benn favours, are the media, many of which have been a good deal more interested in alarming than informing the public.

Both sides claim the Flowers Commission is with them and those who are puzzled by the delicate path trodden by Sir Brian Flowers suggest that he has changed his mind on reprocessing. Perhaps, as Flowers has said, the difficulty lies in the fact that "beginning with an optimistic view of the science and technology of nuclear power in the normal course of events, [the Commission] ended in such a cautionary fashion towards establishing too quickly the major reliance upon nuclear power that the development of commercial fast breeder reactors is intended to facilitate".

There are certainly plenty of questions to which a planning inquiry could address itself, among them the consequences to Windscale of stricter limits on air and water pollution, the reasons for the difficulties that virtually every oxide reprocessing plant in the world has run into, the feasibility of glassification, the question of design to withstand sabotage and the economics of reprocessing. A Planning Inquiry Commission might also consider the disposal of plutonium. Most of these issues were touched upon by the Flowers Commission but not in great detail. The mere fact that such a list of grey areas can so easily be compiled is now seen as perhaps the strongest argument for an inquiry. □

Radiating doubt

Australia's parliamentary debate on the Fox Report ended earlier this month. Peter Pockley reports from Sydney

IF anyone ever needed confirmation that uranium "debates" essentially reflect the politics of power and not the supposedly cool rationality and ultimate certainty of the scientific approach, the current Australian situation provides a nice case study. To the protests of the environmental movement, the Fraser government has announced a decision to allow mining to proceed on some uranium leases to fulfil existing export contracts with West Germany, Japan and United States for a total of 10,700 tonnes of uranium oxide.

This decision was announced within two weeks of the release of the long-awaited *First Report of the Ranger Uranium Environmental Inquiry** under the chairmanship of Mr Justice Russell Fox (hence the popular description "The Fox Report"). Fifteen months of thorough and judiciously fair collection of evidence and viewpoint, and detailed analysis thereof, was concentrated into the report which was commissioned in July 1975 under the Whitlam government's Environment Protection (Impact of Proposals) Act (1974). Three men—Judge Fox, medical Professor Charles Kerr and engineer Graeme Kelleher—were charged with examining a proposal by Ranger Uranium Mining Pty Ltd to recover 85,000 tonnes of U_3O_8 from an area 200 km east of Darwin in Australia's remote Northern Territory.

Because the total "reasonably assured and estimated additional uranium resources" of Australia amount, according to the Inquiry's figures, to about 353,000 tonnes of U_3O_8 —a significant fraction (nearly 10%) of the world total—the Inquiry accepted arguments that Australian decisions on uranium mining should be based on a thorough investigation of the nuclear scene world-wide. Indeed, when Mr Fraser tried to wind up the Inquiry by imposing an early deadline, as he was doing for most Whitlam-initiated inquiries which were in progress when he assumed office, Judge Fox publicly told him to go hang and Fraser had to give in. In retrospect, this was the first sign that there might not have been a close concurrence between the views of the Inquiry and the decisions of a government which is in favour of an expanding mining industry.

The First Report dealt with broad

issues brought to the fore by the discovery of massive uranium resources in several areas of Australia in the last 10 years, particularly in the Northern Territory. Though sparsely populated, much of the Territory is environmentally unique and also has large tracts of "reserves" through which the depressed Aboriginal peoples have special claims on discovered resources. The "findings" of the First Report are couched in generalised terms, with a sprinkling of—effectively—double negatives, which have allowed some differing interpretations by the original protagonists—miners and environmentalists—none of whom give the appearance of altering their original positions after the Report's publication. The Inquiry is now moving on to its next job, that of compiling a technical report on the environmental aspects of the proposed Ranger mine itself, and taking account of the untested effects of a recent Aboriginal Land Rights Bill.

To debate or not to debate?

The government's rapid decision to meet existing contracts, which had been put on ice pending the Fox Report, was clearly predetermined, and took scant regard of the Report's earnest final recommendation. This suggested

that no decision be taken in relation to the foregoing matters (i.e. the mining, milling and sale of uranium) until a reasonable time has elapsed and there has been an opportunity for the usual democratic processes to function, including, in this respect, parliamentary debate.

On announcing the government's decision, the Minister for Environment, Housing and Community Development, Mr Kevin Newman, promised a later debate in Parliament. The media divined that the issues at stake would split the country and both major political parties. The Labor Party and the union movement began to fulfil the prediction by publicly baring their souls, as has been their (destructive) wont in the past. In the event, however, the pragmatists in the Parliamentary Labor Party argued successfully in favour of retaining jobs for miners in the short term, and endorsed the government's green light for limited mining and export of uranium to meet existing contracts.

The increasingly confident uranium lobby—miners, the Atomic Energy Commission and the few nuclear experts in universities—engaged with

some aggression the numerically superior but collectively poorer environmentalists in strident clashes in the media, and the promised debate in Parliament, begun on the last day of November, was a fizz. Only a quarter of the total membership of the House of Representatives bothered to front up—or perhaps many members wanted to avoid being seen to be counted. The only highlight of the "debate" was the rare rebellion of one backbencher in the usually well-disciplined Liberal Party; Mr Don Chipp, a former minister passed over by Mr Fraser, proposed a two-year "moratorium" on uranium mining and export and threatened to cross the floor. The debate was defused, and probably buried, by being adjourned without a vote.

The environmentalists' hopes were then pinned on the union movement, elements of which had threatened withdrawal of labour on key links in the production chain (railway transport of processed ore to the ports, for example). But they were disappointed by the decision, early this month, of the powerful Australian Council of Trade Unions to endorse the government's decision. Thus has a test case, anticipated internationally as being of significance because of the thoroughness and (for uranium matters) rare openness of the Inquiry, become largely uninfluenced by public debate. The Parliamentary and industrial wings of the Labor Party have covered themselves by making strong noises against any extension of the present limited decisions to the sanctioning of uranium mining and export generally without "full public debate" or even a national referendum. But unless Labor wins power in Canberra again, it is virtually certain that the Fraser government will not stage such a debate and decision-making process.

Doubts remain

The Commissioners have become so worried about the government's interpretation of their final words that they are reported to have protested directly to Mr Newman saying that their two main conclusions are "findings" and do not amount to "recommendations" which can be used to justify the government's actions. The Commissioners must now be feeling that the wind of political forces may continue to blow so strongly over their heads as to drown out their voices.

The strongest thing the report seems to have still going for it is its uncompromising approach to the dangers of terrorist activity with fissile material and the inherent weakness of the Non-Proliferation Treaty and safeguards. They believe that proliferation is most likely to originate from fuel reprocessing plants and that "existing safe-

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guards may provide only an illusion of protection". On nuclear theft, the Report says "the risks are real and will tend to increase with the further spread of nuclear technology".

The moral argument against uranium mining and export, applied internationally, which says that Australia should not contribute further to the world's problems, has cut no ice with the government, which paints such protests as being against the national interest measured in terms of overseas earnings and jobs. In response, the environmentalists point to the Report's economic analysis which concluded that the contribution of the potential income from the Ranger deposit to national income is relatively small—only 0.1% in 1980–81, rising to about 0.5% in 1990–91, and declining to 0.4% by the end of the century.

On the question of disposal of radioactive wastes, the Report emphasises that, despite the claims of the nuclear industry, "the whole problem is one of first-rate international importance" and urges that "some internationally acceptable system (be) established for the disposal of high-level wastes, and international supervision of what is done".

Expert witnesses?

The Commissioners made a damaging but probably long overdue attack on the credibility of so-called "expert witnesses", many of whom, it is clear, they heard only with patient sufferance: "In considering the evidence", they say,

we have found that many wildly exaggerated statements are made about the risks and dangers of nuclear energy production by those opposed to it. What has surprised us more is a lack of objectivity in not a few of those in favour of it, including distinguished scientists.

From the published list of witnesses, it is not hard to identify the latter group.

In noting the strong emotional overtones of the evidence, the Commissioners found that "distinguished nuclear scientists" were to be found flatly opposed to each other, but "the final decisions should rest with the ordinary man and not be regarded as the preserve of any group of scientists or experts, however distinguished". And in a further attempt to prick the hardened skins of the nuclear lobby, the Commissioners say that "a few of the publicists for nuclear development characterise their opponents as lobbyists or dissidents, or worse". They go on:

We would wish to make it quite plain that before us the opposition has come from a wide cross-section of the general community, and we would not be prepared to conclude that their motives and methods are any less worthy or proper, or intelli-

Fox findings in full

The Fox Report's findings, reflecting the summarised evidence and its analysis of that evidence, may remain its most lasting contribution to an ever-widening debate. In full, they read as follows:

These findings and recommendations are to be read and understood in the context of the Report as a whole and with particular reference to the sections of the Report in which they are respectively discussed.

1. The hazards of mining and milling uranium, if those activities are properly regulated and controlled, are not such as to justify a decision not to develop Australian uranium mines.
2. The hazards involved in the ordinary operations of nuclear power reactors, if those operations are properly regulated and controlled, are not such as to justify a decision not to mine and sell Australian uranium.
3. The nuclear power industry is unintentionally contributing to an increased risk of nuclear war. This is the most serious hazard associated with the industry. Complete evaluation of the extent of the risk and assessment of what course should be followed to reduce it involve matters of national security and international relations which are beyond the ambit of the Inquiry. We suggest that the questions involved are of such importance that they be resolved by Parliament. In Chapters 15 and 16 we have gone as far as the terms of reference and the evidence permit in examining the courses open and in making suggestions.
4. Any development of Australian uranium mines should be strictly regulated and controlled, for the purposes mentioned in Chapter 16.
5. Any decision about mining for uranium in the Northern Territory should be postponed until the Second Report of this Commission is presented.
6. A decision to mine and sell uranium should not be made unless the Commonwealth Government ensures that the Commonwealth can at any time, on the basis of considerations of the nature discussed in this Report, immediately terminate those activities, permanently, indefinitely or for a specified period.
7. Policy respecting Australian uranium exports, for the time being at least, should be based on a full recognition of the hazards, dangers and problems of and associated with the production of nuclear energy, and should therefore seek to

limit or restrict expansion of that production.

8. No sales of Australian uranium should take place to any country not party to the Non-Proliferation Treaty. Export should be subject to the fullest and most effective safeguards agreements, and be supported by fully adequate back-up agreements applying to the entire civil nuclear industry in the country supplied. Australia should work towards the adoption of this policy by other suppliers.

9. A permanent Uranium Advisory Council, to include adequate representation of the people, should be established immediately to advise the Government, but with a duty also to report at least annually to the Parliament, with regard to the export and use of Australian uranium, having in mind in particular the hazards, dangers and problems of and associated with the production of nuclear energy.

10. The Government should immediately explore what steps it can take to assist in reducing the hazards, dangers and problems of and associated with the production of nuclear energy.

11. Policy with regard to the export of uranium should be the subject of regular review.

12. A national energy policy should be developed and reviewed regularly.

13. Steps should be taken immediately to institute full and energetic programs of research and development into (a) liquid fuels to replace petroleum and (b) energy sources other than fossil fuels and nuclear fission.

14. A program of energy conservation should be instituted nationally.

15. The policy of the Government should take into account the importance to Australia, and the countries of the world, of the position of developing countries concerning energy needs and resources.

Our final recommendation takes account of what we understand to be the policy of the Act under which the Inquiry was instituted. It is simply that there should be ample time for public consideration of this Report, and for debate upon it. We therefore recommend that no decision be taken in relation to the foregoing matters until a reasonable time has elapsed and there has been an opportunity for the usual democratic processes to function, including in this respect, parliamentary debate.

gently conceived, than, in general, are those of the supporters of nuclear development.

Political postscript

The 300 or so people at the Mary Kathleen mine in Queensland, the only active uranium producer at the moment, are dependent on the mine's continuance for employment and are now assured of a job for a few years while the existing contracts are fulfilled. But the investment-led recovery of the national economy, in which the hoped-for expansion of the mining industry played such a key role, has been thrown into confusion since the Fox Report and the decision to export. The massive 17½% devaluation at the

end of November, followed in only 9 days by a 2% revaluation and mostly inconsequential tariff cuts, have rocked the government and its traditional supporters in industry and the press.

The style of Mr Fraser was to purvey a well-controlled image of businessman-like decisiveness, repairing the damage allegedly caused by Mr Whitlam's Labor administration. Suddenly, and by its own hand, the government seems vulnerable. If a uranium debate really gets off the ground in 1977 following the Fox Report No. 2, it is anyone's guess as to whether the government will have things all its own way as it did with Report No. 1. □

news and views

Arrangement of immunoglobulin genes

from Alan R. Williamson

THE complete sequences of the first two light chains of human immunoglobulin determined by Hilschmann and Craig (*Proc. natn. Acad. Sci., U.S.A.*, **53**, 1403–1409; 1965) showed that the unique features of each chain lay in the N-terminal half whereas the C-terminal halves of the two chains were identical. Extensive sequence data from both light and heavy chains have since shown that each chain has a unique N-terminal sequence (variable region) of about 110–120 amino acids. The hypothesis that two genes code for each immunoglobulin chain was advanced by Dreyer and Bennett in 1965 to account for these data, and patterns of inheritance have supported the proposal that a single constant (C) region gene is expressed in conjunction with any one of a set of variable (V) region genes.

The question then arises of the stage at which the information from the V and C genes is combined to produce the complete antibody chain. Evidence against joining at the polypeptide or RNA levels has steadily accumulated. Contiguous translation of V and C regions from the same mRNA (Milstein *et al.*, *Nature*, **252**, 354; 1974) and the absence of V and C scrambling in hybrid cells (Köhler and Milstein, *Nature*, **256**, 495; 1975) both fit more easily the hypothesis that the V and C genes are rearranged and joined together in the genome.

Evidence that the physical arrangement of V and C genes differs in the genome of embryonic and somatic cells has now been presented by Hozumi and Tonegawa (*Proc. natn. Acad. Sci. U.S.A.*, **73**, 3628; 1976). The experiment is elegantly simple in concept; there are however limitations to the interpretation. DNA (either from BALB/c mouse embryos (12 or 13 d old) or from the BALB/c myeloma tumour MOPC321) is specifically fragmented with the restriction endonuclease *Bam*H1. The fragments are separated electrophoretically on slabs of agarose which are then cut into sections and the DNA extracted from each one. Each fraction of DNA is tested for sequence complementarity to κ -chain mRNA prepared from MOPC321 tumour cells.

Different patterns of complementary DNA fractions were found with embryonic and tumour DNA. In a digest of embryonic DNA, two fragments (about 6.6 and 9.2 kb) showed significant hybridisation with the κ 321 mRNA probe whereas a digest of tumour DNA showed significant hybridisation to only one fragment (about 3.5 kb). The peaks of hybridisation are broad and only a fraction of input radioactivity was hybridised (<20%). Identification of the peaks is made using a second probe specific mainly for the C region of κ chain. This probe was prepared after the fragmentation of mRNA during iodination by selection of the appropriately sized 3'-end fragments containing poly(A). By this test the 9.2 kb fragment of embryonic DNA contains C_K sequences but no V_{321} sequence whereas the 6.6 kb fragment contains V_{321} sequence but no C_K sequence. The 3.5 kb fragment of tumour DNA apparently contains both C_K and V_{321} sequences. These results are interpreted by the authors as showing that V_{321} and C_K genes are located separately in embryonic DNA but occur in the same short stretch of 321 tumour DNA. Clearly this interpretation is consistent with somatic rearrangement of V_{321} and C_K to give a contiguous gene. Alternative explanations involving two short stretches of DNA in 321 (one V_{321} and one C_K) or mutations introducing and removing *Bam*H1 sites are considered less probable than the contiguous gene hypothesis.

It is tempting to accept that somatic rearrangement of V and C genes is now beyond reasonable doubt and to seek for some mechanism. But it is when one comes to consider the possible mechanisms that the present data appear too simple in the light of some of the other evidence available from studies of immunoglobulin genes in myeloma cells.

Immunoglobulin-bearing cells are functionally haploid (that is, only one allele is expressed). The restriction mapping, which results in only one type of fragment homologous to the V or C probes in the tumour cell, suggests therefore that either MOPC321 cells are physically haploid or that some form of homozygosis has occurred yielding homologous

chromosomes with the same VC pair on both. There is also the complication that myeloma cells are usually aneuploid but the authors do not comment on this point. The C_K gene seems to be in a linkage group on chromosome 6 in the mouse, so knowledge of the number of copies of this chromosome in 321 tumour cells would clarify the interpretation.

Studies of somatic mutation in myeloma proteins suggest that myeloma cells have only one expressed VC_K gene (Milstein *et al.*, in *Molecular Approaches to Immunology*, edit. by Smith, E. E., and Ribbons, D. S., 131–148, Academic, New York and London, 1975) although some myeloma tumours possibly express an additional C_K gene (Kuehl *et al.*, *Cell*, **5**, 139; 1975) and there is also evidence for more than one C_K gene per haploid genome (Stavnezer *et al.*, **88**, 43; 1974). The absence of C_K restriction fragments in myeloma DNA is therefore puzzling and a single $V_{321}C_K$ restriction fragment may be too simple an interpretation of the data. Alternatively MOPC321 may be an unusual tumour which has lost all but the functional C_K gene.

A different way of asking the rearrangement question would be to map the DNA of a tumour other than 321 (that is, not expressing V_{321}) using the V_{321} mRNA probe. Analysis of 321 tumour DNA with another κ mRNA would also be valuable. In these experiments the relative locations of expressed and non-expressed V region sequences could be determined on the same DNA digest.

Hozumi and Tonegawa comment that their choice of hybridisation technique was limited by the purity of κ mRNA currently achievable. Mach and Rougeon reported at the 9th Harden Conference the successful cloning of κ cDNA in a bacterial plasmid (see *News and Views*, **263**, 726; 1976). The availability of homogeneous nucleic acid probes will make possible the application of hybridisation techniques using excess probe; even 90% purity of probe is insufficient for such techniques.

Conclusive evidence for somatic rearrangement of antibody genes may not be far away. Knowledge of the mechanism is probably a little more remote. □

Regulating steroid action of the molecular level

from Robert Shields

EVERY budding endocrinologist learns the current dogma of steroid action at his Alma Mater's knee. The patter runs something like this. After entering the cell steroids bind to specific receptors (present only in responsive cells) which migrate to the nucleus, switch on specific genes and so increase cytoplasmic mRNAs coding for specific proteins. While there is good evidence for the first and last parts of the scheme, what happens in the nucleus is still confused.

It is clear in some cases at least that the steroid hormones can be very selective in how many of the cell's 1×10^4 – 3×10^4 different mRNA species they control. For instance, in the *Drosophila* polytene chromosomes the pattern of gene puffing induced by ecdysone shows that this steroid hormone controls relatively few genes (Ashburner *et al.*, *Cold Spring Harbor Symp. quant. Biol.*, **38**, 655; 1973). In other systems direct measurements of mRNA species suggest that only a limited number of sequences are steroid regulated. Experiments using DNA complementary to purified ovalbumin mRNA have shown that oestrogen induction of ovalbumin in the chick oviduct involves an enormous increase in ovalbumin mRNA (McKnight *et al.*, *J. biol. Chem.*, **250**, 8105; 1975; Harris *et al.*, *Biochemistry*, **14**, 2072; 1975) and less direct experiments suggest that the mRNA for conalbumin also increases (Palmiter *et al.*, *Cell*, **8**, 557; 1976). But these experiments do not indicate how many other proteins are affected by oestrogens.

An approach to this problem is to analyse the mRNA complexity in tissues of different hormonal status by measuring the kinetics of hybridisation of total mRNA to its complementary DNA. Hybridisation of excess mRNA isolated from hormone-withdrawn or oestrogen-stimulated chick oviduct to its respective complementary DNA showed that oestrogen treatment led to the synthesis of many thousands of copies of between one and ten mRNA species (Axel *et al.*, *Cell*, **7**, 247; 1976; Monahan *et al.*, *J. biol. Chem.*, **251**, 3738; 1976). This handful of species includes ovalbumin mRNA (Axel *et al. op. cit.*) and presumably the mRNA species for other egg white proteins as well.

Within the margin of error of this type of homologous hybridisation it is impossible to state definitely whether oestrogens induce significant numbers of other mRNA species that are

normally present in only a few copies per cell (low abundance). Heterologous hybridisations using mRNA isolated from oviduct in one hormonal state and cDNA prepared from mRNAs from another hormonal state should be able to decide this question. Where such heterologous hybridisations have been done (rat prostate) the major effect of the steroid hormone (testosterone) seems to be the increase in the number of copies of relatively few mRNA species (M. Parker, personal communication). Further support for the very limited action of steroids comes from experiments where the total proteins synthesised in glucocorticoid-treated hepatoma (HTC cells) were examined by two-dimensional electrophoresis. Here again it is apparent that only a handful of the cell's proteins including that old stalwart tyrosine transaminase are affected by the steroid (R. Ivarie and P. O'Farrel, personal communication). In all this it should not be overlooked that while steroids may alter the relative synthesis of only a few mRNAs they may also exert general effects on the total amount of RNA present in the cell.

How does the receptor-steroid complex bring about these relatively specific changes in a few mRNAs? By analogy with prokaryotes, in which gene control is understood rather better, receptors could bind to specific regions on the eukaryotic chromatin similar to a bacterial operator leading to increased gene transcription. The analogy would demand that the nuclear gene control sites would be limited in number (comparable to the number of genes controlled by the steroid) and the binding of receptor to these sites would be specific (that is, it would have a higher affinity for control than non-control regions of the genome). Furthermore, any explanation of steroid regulation should account for the fact that there are around 10^4 receptors per cell (although only 10 or fewer genes are steroid regulated).

Measurements of the numbers of nuclear binding sites for receptors have provided conflicting results. *In vivo* there is a good correlation between biological effect, steroid receptor binding and transfer of the receptor steroid complex to the nucleus (Katzenellenbogen and Gorski, *J. biol. Chem.*, **247**, 1299; 1972). The proportion of cellular steroid complexes in the nucleus is, however, independent of the dose of administered steroid (Williams and Gorski, *Proc. natn. Acad. Sci. U.S.A.*, **69**, 3464; 1972), a result that is incon-

sistent with the presence of a strictly limited number of nuclear binding sites for the receptor. Instead the results point to at least a 5–10-fold excess of nuclear binding sites over steroid receptors, approaching 10^5 sites per cell, far exceeding the number of genes under steroid control. As yet there are only a few eukaryotic mutants that are of much use in dissecting the steroid-mediated events in the cell nucleus so attention has been focused on the interaction *in vitro* of steroid receptors and isolated nuclei of nuclear subfractions. While a great many results show that the number of nuclear binding sites for receptor may be very large (Chamness *et al.*, *Biochemistry*, **13**, 327; 1974; Simons *et al.*, *J. biol. Chem.*, **251**, 339; 1976) several other *in vitro* experiments suggest that this is not the case (Higgins *et al.*, *J. biol. Chem.*, **248**, 5866; 1973; Rousseau *et al.*, *J. Steroid Biochem.*, **5**, 935; 1974). Even if the lowest number for nuclear binding sites is accepted ($\sim 15,000$) this is at least two orders of magnitude greater than the number of controlled genes.

One way out of this dilemma has been suggested recently (Yamamoto and Alberts, *Cell*, **4**, 310; 1975). Their proposal is that steroid-receptor complexes bind with low affinity to a very large number of nonspecific sites in the cell DNA. This nonspecific binding is the binding that is measured in *in vitro* experiments and completely masks the binding of receptor to up to 10^3 specific sites of high affinity which are the true control regions. The model is strongly influenced by studies of the *lac* operon in *E. coli*. Here it was known on genetic grounds that the repressor bound to gene control sites but specific repressor binding could not be demonstrated to *E. coli* DNA. Only when the *lac* operon was purified by incorporation in phage could specific repressor binding be demonstrated (Lin and Riggs, *J. molec. Biol.*, **72**, 671; 1972). This theory also neatly accounts for the apparently large number of receptors in the cells. They are present in large quantities so that enough receptors are left to bind to control loci when the rest are mopped up by binding to nonspecific regions. Perhaps the nonspecific binding to multiple sites is the biological price that has to be paid to get reversible receptor binding to a few specific control elements. But, if the measured receptor binding sites (the nonspecific sites) consist only of DNA as has been suggested (Yamamoto and Alberts, *op. cit.*) there should be no specificity of receptor binding to tar-

get and non-target nuclei. Reports on this point are conflicting, both tissue specificity (Higgins *et al.*, *J. biol. Chem.*, **248**, 5873; 1973) and lack of specificity (Chamness, *op. cit.*) have been reported.

Perhaps the situation will only be finally resolved when purified receptor is available and it is possible to clone the DNA fragments containing the controlled gene and its operator region and so study receptor binding *in vitro*. Two systems are presently being explored in this manner, the control of ovalbumin synthesis in the oviduct (Woo *et al.*, *J. biol. Chem.*, **251**, 3868; 1976) and the glucocorticoid regulation of mouse mammary tumour virus (see *News and Views*, **259**, 193; 1976). □

Fish schooling

from John Krebs

Two recent papers have shed interesting new light on fish schools. Many species of fish swim in polarised and coordinated groups containing anything from half a dozen to many thousands of individuals, according to the species. Pitcher *et al.* (*Science*, **194**, 963; 1976) have investigated the sensory mechanisms involved in schooling, and Breder (*Fish Bull.*, **74**, 471; 1976) has suggested a new functional explanation for the schooling habit.

In the past, several workers have emphasised the importance of vision in schooling: schools of various species tend to break up at low light intensities. Pitcher *et al.* studied the schooling abilities of Saithe (*Pollachius virens*) fitted with black plastic eye-cups. These temporarily blind fish could be scooped out of the water showing no attempt to avoid the net, but could still join and maintain their position in a fish school swimming around a large circular tank. The blind fish showed some small differences in behaviour: they moved from side to side within the school more than their companions, but in the Saithe at least, vision is clearly not necessary for schooling. Pitcher *et al.* go on to show that if the lateral line system of pressure detectors is cut, the blind fish no longer school, which seems to show that the blind Saithe use water movements created by their companions in order to stay in position in the school.

The adaptive significance of schooling is also much debated and several hypotheses (not mutually exclusive) have emerged. One is that schools are an anti-predator adaptation, the idea being that the individuals in a school "seek cover" from potential attackers by hiding behind a fellow schooler

(Hamilton, *J. theor. Biol.*, **31**, 295; 1971). This might be called the "after you" principle. Experiments have shown that predators also tend to be confused when faced with a milling school of prey, so that attack success goes down (Neill and Cullen, *J. Zool. Lond.*, **172**, 549; 1974). Another possible benefit of being in a school is that each individual is able to parasitise (by copying) the success of its companions in finding good feeding places: an effect well known to fishermen and blackberry pickers. A less well-documented possibility is that there may be a hydrodynamic, energy saving, advantage of schools. As a result of its swimming movements, each fish produces a series of vortices in the water to either side of its body, and if a following fish swims in exactly the right spot, it could in theory get a forward boost from the vortex, allowing it to free wheel and save energy (Weihs, *Nature*, **241**, 290; 1973). Only the hapless individuals at the front gain nothing, but fish are known to jostle for positions at the back and centre of a school, and this hydrodynamic theory may explain why. Breder also suggests a hydrodynamic advantage which would benefit all but the front swimmers. He notes that the mucus produced by the bodies of fish has an important effect in reducing frictional drag. In some species even a 5% solution of mucus can reduce drag by up to 60%. Breder's idea is that fish in a school (except for the front swimmer) benefit not only from their own mucus, but from the slime washing off the bodies in front of them. He suggests that schooling may have originated simply as a consequence of fish finding it energetically easier to swim along behind someone else.

Breder's suggestion is an addition rather than an alternative to the anti-predator and feeding advantages of schooling. In fact, to explain the differences between species of fish in their tendency to form schools, one will have to look for ecological factors such as the risk of predator attacks, since the hydrodynamic advantage probably would apply roughly equally to all fish. □

Troubled waters

from Peter D. Moore

THE art of compromise may not be dead, but it is certainly not thriving, judging by the views put forward at a conference (Recreation and Conservation in Water Areas) held by the Royal Society of Arts in London on November 22. The meeting succeeded in demonstrating that there is little common ground, or water, between the

two interests. Such a state of affairs is predictable, for bird watchers and their ilk have never been noted for their affection towards water-skiers and power-boat drivers, but what does seem remarkable is the total lack of understanding which each group of water-users has of the others interests and requirements. The problem is a very real one for the regional Water Authorities, which were set up under the 1973 Water Act. This same Act makes these Authorities responsible for making their waters and associated lands available for public recreation (Section 20) and also places statutory obligation upon them to grant public access and to have regard to conservation interests (Section 22). Whether they like it or not, the Water Authorities must try to satisfy all demands.

B. Hackett (University of Newcastle-upon-Tyne) represented the landscape designer's point of view and evidently found considerable aesthetic appeal in the geometrical, concrete-edged lake within a built landscape setting. Many may share his view, but he cannot expect enthusiasm from the angler or the wildlife conservationist; neither can he expect these groups to be responsive to his 'semi-natural' compromise, which is a body of water surrounded by carefully selected shrubs and neatly planted flower beds. For the planner, I suppose, nature can only be attractive when planned.

Then there is the true recreationist. Does N. R. Collins (Sports Council) really believe that the disturbance created by water skiers reoxygenates water, or was that a joke? Even if it were demonstrably true I cannot imagine the three million odd British anglers being very impressed. There is no chance of compromise here.

C. Newbold (Nature Conservancy Council) presented a distinctly unpromising statement of the conservationist's position. Quoting the work of Hume (*Br. Birds*, **69**, 178; 1976) he explained that a power boat puts diving ducks (the goldeneye for example) to flight at a range of 500–700 m, sailing boats do so at 350–400 m and people on the bank at 100–200 m. He rather assumed that the latter would be anglers, but they could equally well be bird watchers themselves. The figures do, however, serve to highlight the conflict of interests between bird watchers and just about all other water users. The vociferous and adverse audience reaction to Newbold's suggestion that the construction of a restaurant on the shores of the Loch Insh Nature Reserve was a retrograde step in terms of amenity, ably demonstrates that 'amenity' can mean different things to different people.

Wildfowling has had much experience in respecting the needs of others

and J. Harrison (Wildfowlers' Association of Great Britain and Ireland) presented a broad and sympathetic survey of the problems of water use. Anglers and bird watchers can be reconciled, he claimed, if the anglers are restricted to specifically designed sites on the bank, screened by trees and bushes. I wonder how the anglers react to such a screen when trying to make a cast.

Reconciliation is still a long way off. Zoning of the larger lakes, such as Loch Lomond and Llyn Tegid in which certain activities are restricted to specific areas, is successful at some sites, but requires unselfish cooperation from all water users. Smaller sites may have to be reserved for single activities. This will still create arguments over priorities, for England and Wales contain only 300,000 acres of water space and conservationists assure us that 94% of southern grade 1 and grade 2 conservation sites are currently used for recreation. □

Tsetse ecology and behaviour

from a Correspondent

A Study Workshop on the ecology and behaviour of tsetse flies was held in Nairobi on September 28–October 2, 1976. It was organised by Professor T. R. Odhiambo, Director of the International Centre of Insect Physiology and Ecology, Nairobi.

THE objectives of the workshop were to assess the state of current knowledge in this field, and on that basis to formulate plans for future research with specific reference to the possible development of acceptable methods of controlling the tsetse vector of trypanosomiasis.

Scientific sessions opened with a review of current knowledge of tsetse ecology by A. M. Jordan (University of Bristol), who emphasised the need for a quantitative appraisal of mortality factors operative in natural tsetse populations. This was followed by accounts of recent work on the ecology of an atypical, peridomestic populations of *Glossina palpalis* in lower Zaire (P. van Wettere, WHO, Upper Volta) and of *G. tachinoides* in West Africa (D. A. T. Baldry, WHO, Geneva); and of typical populations of *G. swynnertoni* in Tanzania (S. K. Moloo, ILRAD, Nairobi), of *G. morsitans morsitans* in Zambia (S. N. Okiwelu and L. C. Madubunyi (University

of Nigeria, Nsukka)) and in Botswana (R. Allsopp, COPR, London), and of *G. pallidipes* in Kenya (J. van Etten, ICIPE). A dominant theme throughout was the possible occurrence of genetically distinct populations within a species, characterised by differences in host preference and other aspects of behaviour which might affect vectorial capacity. With regard to the peridomestic populations which could assume increasing importance in the context of developing Africa, the possibility of reducing challenge by appropriate modifications of livestock management practice, or by limited but highly selective application of insecticides, should clearly be investigated.

Recent developments in tsetse genetics were reviewed by W. Helle (Universitat van Amsterdam), who concluded that empirical studies of tsetse genetics would have an important part to play in the assessment of possible genetic differences between populations, and in guarding against the occurrence of undesirable genetic drift in colonies destined for use in sterile-male release programmes (such as the current Tanga project, which was later described by the project leader, L. Williamson (Tsetse Research Project, Tanga, Tanzania)). L. P. S. van der Geest (Universitat van Amsterdam) presented preliminary results of his isoenzyme investigations, reporting that different laboratory colonies could be shown to differ substantially in the relative frequency of alleles at the leucine-alanine peptidase locus. The importance of extending studies of this kind to natural populations of tsetse flies was agreed.

The session on tsetse behaviour was opened by J. Brady (Imperial College, London), who reviewed work on endogenous rhythms in tsetse flies, and described an analysis of available field data on diurnal patterns of activity in terms of an interplay between endogenous and environmental factors, emphasising the importance of a close integration of field and laboratory investigations. The need for a close study of the "medium range" reactions of tsetse flies to their hosts (as opposed to the long-range olfactory and the short-range thermal and gustatory reactions) was brought out; this, too, was the main theme of G. A. Vale (Department of Veterinary Services, Salisbury, Rhodesia) in his discussion of sampling techniques, where the effectiveness of different sampling methods for different components of the tsetse population and for different species was shown to be closely related to this aspect of behaviour. Vale also reported that investigations were in progress to explore the possibility of using the long-range attraction of tsetse flies to host odours as a basis for a sterile-male

release programme based on the capture, rather than on the rearing, of male flies.

A new dimension was introduced by J. Keiding (Danish Pest Infestation Laboratory, Lyngby), who described aspects of the biology of *Musca domestica* in Denmark, and showed that many features of its biology had been capable of convincing interpretation on the basis of results obtained from a range of relatively simple laboratory experiments. It was agreed that similar work could usefully be carried out with the newly emerged tsetse fly, which might represent a vulnerable stage in the life history. In the subsequent discussion, it was emphasised that in studies of tsetse behaviour due attention should be paid to the possible importance of group or social interactions.

A. Youdeowei (University of Ibadan) presented a paper on the salivation behaviour of the tsetse, based on a newly developed experimental technique, and the possibility was mentioned of interfering with trypanosome transmission at this stage of the cycle. This was followed by a discussion by T. Jaenson (University of Nairobi) of his recent work on the mating of *G. pallidipes*, which has long been recognised as a difficult species from the point of view of laboratory maintenance because of its recalcitrance to laboratory mating. The increased receptivity which characterised the first week in the life of adult females was shown to be associated with a corre-



A hundred years ago

A MEETING was recently held in Birmingham of the Council in connection with the projected aquarium for that town. We are glad to see that the arrangements for carrying the scheme into execution are well forwarded, and a Committee was appointed at the meeting to make all necessary preliminary arrangements. The proposed plan of the aquarium seems to us all that could be desired, and we are glad to see that Mr. Hughes, and other speakers at the above meeting, showed a laudable desire to make the institution serve important educational purposes; we hope, at least, that it will not degenerate into a second-rate music-hall and miscellaneous rendezvous.

From *Nature*, 15, December 21, 1876; 1876.

sponding decrease in copulation time; additionally, evidence in support of the existence of genotypically distinct populations of the species was adduced on the basis of differences in copulation time between colonies deriving from different localities. E. Bursell (University of Rhodesia, Salisbury), described recent work on the metabolic basis of flight activity in the tsetse, and presented a source/sink analysis of energy reserves on the basis of which it was concluded that male tsetse flies might spend far more time in flight than had hitherto been supposed, whereas values for the female raised the possibility that slight reductions in host availability might cause a substantial decrease in reproductive potential.

In reviewing the proceedings as a whole, participants agreed that the potential of host odours and pheromones for use in control programmes and in the development of sampling techniques for low density populations deserved careful investigation. They noted, further, that a constantly recurring theme throughout the deliberations had been the need to mount a large-scale, long-term, multidisciplinary investigation of the crucial relation between the tsetse and its hosts in the natural environment, with particular reference to the quantitative assessment of mortality factors operating on a natural tsetse population. Only in this way could a basis be provided for the better understanding, in quantitative terms, of factors governing the population dynamics of the species, without which the potential for the development of integrated control programmes would be incapable of proper assessment. In this connection it was emphasised that the activity of tsetse control should not be allowed to take on "a life of its own", but that it should be seen as just one element in the broader context of the proper management of natural resources. □

Ecdysone binding across the Rhine

from David Whitehead

An informal Ecdysone Workshop was held at the Royal Entomological Society, London on November 11, 1976.

ECDYSTERONE, it is understood, elicits its manifold response by entering the cells of the target tissue whereupon it is carried to the nucleus by specific cytosol proteins where it may or may not interact with a DNA repressor on

the genome. Whereas the evidence for this type of model may now be good for mammalian steroid hormones, invertebrate biochemists are just beginning to make headway again.

At the meeting laboratories in Germany and France revealed their up-to-the-minute findings concerning the specific binding of the 'ecdysteroids' ecdysone and ecdysterone (the arthropod moult hormones) to various tissues of a locust (J. A. Hoffman, Université Louis Pasteur, Strasbourg), a crayfish (K-D. Spindler, Technische Hochschule Darmstadt) and to fruit fly salivary glands (H. Emmerich, Darmstadt).

Hoffmann and colleagues have found 'receptors' for ecdysterone in epidermis, fat body and even muscle. Maximal binding was achieved at 10^{-7} M, which happens to be the maximum titre in *Locusta migratoria* as detected by bioassay and by the radioimmunoassay developed in Marseilles (de Reggi *et al.*, *Biochem. biophys. Res. Commun.*, **66**, 1807; 1975). Specific binding of ecdysone occurred in the epidermis only. This supports the contention (P. Karlson and J. Koolman, Philipps Universität, Marburg) that ecdysone is not just a prohormone but is an active primary messenger in its own right. Difficulties have arisen, however, in quantifying binding to the various 'receptors' because the parent tissues were derived from locusts with high endogenous titres of ecdysteroids. Unfortunately, prothoracic gland extirpation or selective X irradiation of larvae block the activation of the binding sites themselves. Therefore, the task of characterising the 'receptors' is necessarily a slow one; for instance, the exact amount of hormone in the nuclei and in the cytosol must be known for each tissue used.

Saturable 'cytoplasmic receptors' for ecdysterone ($\sim 140,000$ daltons) were demonstrated in hypodermis, hindgut and gonads of *Orconectes limosus* using equilibrium dialysis, charcoal assays and equilibrium density gradient centrifugation (Kuppert and Spindler). After partial purification, binding molecules with high affinity for ecdysterone were found in the cytoplasm of salivary glands from mid stage III larvae of *Drosophila* (Beckers and Emmerich). Outside the target cells R. Lafont and coworkers (Ecole Normale Supérieure, Paris) suggested that ecdysteroids could be bound reversibly to small polar components in haemolymph which bore a striking resemblance to the fat body factor of G. Bergstrom and H. Oberlander (*J. Insect Physiol.*, **21**, 39; 1975).

H. Rees (University of Liverpool) reported that ecdysone C-20 hydroxylase from *Schistocerca* Malpighian tubule mitochondria is a mixed func-

tion oxidase involving P-450 cytochrome. Peak activity of the enzyme is reached just before the maximum hormone titre in the 5th instar larva implying that the hydroxylase might regulate the ecdysterone level. Koolman was concerned that metabolism of both ecdysone and ecdysterone into the 3-dehydro derivatives (followed by epimerisation) and into the polar conjugates (glucuronides, glucosides, sulphates) could effectively regulate the titre of moulting hormone in blowflies and locusts. There were, however, demonstrable differences in the activity of the various enzymes between species and even between stages of the same insect. For ecdysone oxidase, a cytosol enzyme in *Calliphora* larvae, Koolman showed that three factors regulate the level of hormone: the enzyme titre, its availability (compartmentation in the cell) and its kinetic properties. (Since the meeting new evidence has come to light that 3-dehydroecdysone—the product of ecdysone oxidation—may be active itself in some systems. It initiated puffing in *Drosophila* salivary glands (G. Richards, University of Cambridge) and yet it did not cross react with rabbit antisera to ecdysone carboxymethylloxime-BSA complex (D. Whitehead, University of Bristol)).

Richards presented evidence of ecdysone-induced chromosomal puffing in the salivary glands of *D. melanogaster* using *in vitro* analysis. He suggested that ecdysteroid titres may fluctuate rapidly in normal development. In particular, in the 12–14 h of the prepupal stage, effective titres of ecdysterone may change from 10^{-7} M to 10^{-9} M and back to 10^{-7} M in the salivary gland. Although radioimmunoassay has recently allowed an investigation of hormone titres previously not possible in fruit flies, total values found may be misleading since biologically inactive ecdysteroids could cross react in the system. For instance, the high titres found *in vivo* 3–8 h after puparium formation would inhibit the normal development *in vitro* of the salivary gland at this mid-prepupal stage. His suggestion was that either the hormone is sequestered *in vivo* so as to reduce the effective titre or it is metabolised to biologically inactive products.

Puparium formation of *D. lebanonensis* is under the control of a circadian oscillation. J. Eeken (Nijmegen, Holland) showed that this oscillation allows pupariation to occur only during specific 'gate' periods every 24 h (L:D=12:12). Injection of a group of larvae with ecdysterone before the 'gate' period which preceded pupariation by 24 h, induces specific puffing in the polytene chromosomes of salivary glands. Injection after the 'gate' is

also followed by puffing but not by pupariation. The result is not due to supplementation of endogenous titres by injection because no ecdysteroids are detectable using the new antisera (Horn *et al.*, *J. Insect Physiol.*, **22**, 901; 1976) before or immediately after the 'gate' period. Hormone titre only increases when pupariation commences where the level is $0.11\text{--}0.15\text{ nmol g}^{-1}$. Similarly Whitehead found that stage III tsetse fly larvae are devoid of hormone during sclerotisation of the poly-pneustic lobes *in utero* as well as during and after birth. The radioimmuno-assay first detected ecdysteroids during pupariation and the titre rose eventually to $0.8\text{--}0.9\text{ nmol g}^{-1}$ after pupal-adult apolysis.

A more practical observation was that 10^{-3} M ecdysterone and inokosterone added to blood fed through a membrane to *Glossina morsitans* females after ovulation caused 55–80% of the eggs and larvae to be aborted. The systemic action may be at the level of the Malpighian tubules (which were activated *in vitro* to secrete urine (Gee, Koolman and Whitehead)) and the uterine glands which nourish the larva, rather than directly on the offspring.

P. Berreur and coworkers (CNRS, Gif sur Yvette, France) demonstrated a simple device for positioning blowfly larvae before ablation of the ring gland complex containing the gland responsible for ecdysone synthesis. Twelve days after the operation the permanent larvae which result contained 0.08 nmol g^{-1} of ecdysteroids whereas the titre at pupariation was normally ten times this level. Furthermore, wing disks of the ablated larvae were unable to incorporate ^3H -uridine normally after injection into the larva. □

Parasite invasion

from V. R. Southgate

The fifteenth meeting of the British Society of Parasitology, entitled Parasite Invasion was held at the Zoological Society of London on October 29, 1976.

PARASITES must, at some stage in their life-cycle, move from one host to another, then find appropriate sites within the host to which they become morphologically, physiologically and biochemically adapted. To do this often requires penetration. Penetration mechanisms of parasites are relatively poorly understood, although recent advances have revealed glimpses of the levels of complexity involved. Therefore it seemed appropriate to review

current knowledge and ideas on the mechanisms of parasite invasion and the accompanying physiological changes which take place. It is of fundamental importance to understand these diverse processes which parasites, in order to be successful, have evolved in association with their hosts.

Parasites attach to host cells/tissues in many ways, and the degree of specificity controlling attachments and invasion varies. For example, P. L. Long (Houghton Poultry Research Station) and C. A. Speer (University of Montana) reported that sporozoites of coccidia will enter a wide variety of cell types *in vitro*: however, *in vivo* studies demonstrate that site specificity is strong although factors influencing this are unknown. In contrast, L. H. Bannister (Department of Biology and Anatomy, Guy's Hospital) reported that the merozoite of *Plasmodium* will adhere to several cell types, but penetration only occurs when the apical surface of the merozoite contacts a red blood cell membrane. Thus, invasion depends upon the recognition of specific receptors, and interest has been aroused in the possible relationships of the receptors of *P. vivax* and *P. knowlesi* with blood group antigens of the Duffy class (Miller and Carter, *Expl Parasitol.*, **40**, 132; 1976). The signal for invasion remains elusive. Different groups of viruses have assorted methods for cell attachment and entry: for example, D. A. J. Tyrrell (Clinical Research Centre, Harrow) indicated that influenza viruses attach to cells by 'interlocking' viral glycoproteins to host cell neuraminic acid residues. If attached to cells which are unsuitable for penetration, these viruses are able to detach themselves by secreting neuraminidase. Helminths perceive certain stimuli through complex sensory receptors which apparently initiate behavioural patterns such as release of cytolytic secretion. The schistosome cercariae which invade man respond to rise in temperature and fatty acids of the host skin (Schiff *et al.*, *J. Parasitol.*, **58**, 476; 1976).

Thus, evidence is increasing to suggest a molecular basis for attachment and signals for invasion, which in turn is helping to explain specificity.

What are the actual mechanics of invasion? Our knowledge of the processes involved are limited, especially in the protozoa. It seems likely that well-defined cell organelles are important. Cinemicrographic studies suggest invasion is an active process on the part of the sporozoite of coccidia, and the conoid (a truncated hollow cone of 6–8 spirally arranged fibrils) is capable of protrusion and contraction. The merozoite of *Plasmodium* somehow induces the host cell membrane to in-

vaginate: the invagination 'sucks' the merozoite inwards. Possibly, substances secreted from the rhoptries and micronemes (merozoite organelles) are absorbed into the host cell membrane, thereby altering its biophysical properties. B. E. Matthews (University of Wales, Bangor) demonstrated that helminths pass through tissue barriers using one or a combination of three methods: mechanical invasion; the use of cytolytic secretions; or normal processes, such as feeding.

Why are there alterations in metabolic pathways after invasion? J. Barrett (University of Wales, Aberystwyth) considered two factors to be of major importance: the metabolism of infective helminth larvae is geared to survival whereas that of adults is directed towards reproduction. Also, the physical environment is different, for example, in homeotherms, the temperature and partial pressure of CO_2 rises, and the partial pressure of O_2 falls. Basically, the metabolism of infective larvae is aerobic whilst that of adult parasites is anaerobic—the switch from one to another is controlled genetically and by physical factors. P. I. Trigg (National Institute of Medical Research, Mill Hill) and W. Gutteridge (University of Kent) argued that difficulties associated with *in vitro* culture and separation of protozoan parasites from their host cells for biochemical analysis are the two main reasons for the incomplete picture of physiological and biochemical changes during life-cycles. These problems are not so acute with the extracellular trypanosomes. Consequently, the morphological and metabolic differences between the vertebrate blood and insect midgut forms are comparatively well-documented. Interpretation of the physiological and biochemical changes in coccidia and *Plasmodium* have been based largely on ultrastructural and cytochemical observations, but the recent advances in the continuous cultivation of *P. falciparum* by Trager and Jensen (*Science*, **193**, 673; 1976) and Haynes *et al.* (*Nature*, **263**, 767; 1976) may help to rectify this imbalance.

Advances in techniques in the future will enable a more precise identification of specific receptors at the molecular level on both parasite and host to be attempted.

Parasitologists will also be trying to uncover the nature of signals which initiate behavioural patterns. Advances in *in vitro* culture of protozoan parasites and use of natural barriers *in vitro* for infective larvae will make collection of material for biochemical analyses easier, but problems of clean separation of intracellular protozoa from host cells will have to be overcome. □

review article

Selective stabilisation of developing synapses as a mechanism for the specification of neuronal networks

Jean-Pierre Changeux & Antoine Danchin*

The specificity of synapses in the mammalian brain cannot possibly be accounted for by one-to-one biochemical matching. The alternative proposed in this article is that connections are genetically specified between classes of cells, but the final wiring pattern depends on the refinement of those collections by selective stabilisation during neuronal activity.

In his 1951 Ferrier lecture¹, J. Z. Young proposed that the mature synapse represents the end product of a continuous process of growth and degeneration of the terminal part of nerve fibres and further suggested that the "modification of such daily growth by functional activity would provide the basis of the plasticity of the nervous system". Since then, the portrait of the synapse has been drawn in more detail with the addition, in particular, of its fine organisation², the elementary mechanisms of its activity^{3,4}, the chemistry of some fine critical components⁵ and the role of factors which selectively affect the growth of nerve terminals⁶. In parallel, and independently, the basic structural features of the genetic material and of its expression have emerged. Encouraged by the success of genetic analysis in the acquisition of this knowledge, molecular biologists interested in the nervous system have focused primarily on its genetic determination^{7,8} and have thus raised the question of how such determination takes place. The DNA present in the nucleus of the fertilised egg may, at most, code for only a few million proteins and this quantity is practically the same in mouse, chimpanzee and man, moreover a significant fraction of it (more than 60%) does not correspond to genuine structural genes⁹. How is it then possible to engender the formidable complexity of the nervous system from such a limited number of genes?

The answer must lie in the mechanics of embryonic development. Models have been proposed which, from the combination of a small number of genes expressing themselves sequentially, and a few yes-no signals, produce a significant diversity of cell types¹⁰. They offer plausible, but still theoretical, explanations for the setting out, for example, of the neuronal somas. The establishment of adult connectivity, particularly of the vertebrate nervous system, is of a higher order of complexity. The call for "gene saving" mechanisms becomes pressing. The hypotheses which have been put forward to explain how nerve cells become interconnected during development differ chiefly on the question of whether the functional activity of the developing network plays a part. Schematically, the two main conceptual attitudes are the following.

(1) For Sperry¹¹ ("cytodifferentiation" or "chemoaffinity" hypothesis) the nerve fibres in the process of growth bear chemical labels complementary to those of their neuronal target; each label reflects the position of a given cell within a set. The pre- and postsynaptic partners recognise and assemble

themselves with a selectivity which depends on the complementarity between cell surfaces. Gaze and Keating¹² and Prestidge and Willshaw¹³ have amplified and extended this cytodifferentiation hypothesis introducing, in particular, a graded affinity between axons and postsynaptic targets and a competition between ingrowing nerve terminals and their postsynaptic partners, both being present in limited numbers. Gaze¹⁴ and Jacobson¹⁵ have also considered temporal factors in the establishment of connections. The differential growth of nerve fibres would be instrumental in bringing an order: reaching their target neurones at different times, the nerve terminals would "number" them in sequential order ("timing" hypothesis). The plausibility of these hypotheses has already been discussed^{12-15,16}.

(2) Since Ramon y Cajal¹⁷ and others, three attitudes have been adopted; briefly they are the following (Fig. 1).

First, as in the "preformist" views just mentioned, the neuronal network is assumed to be specified before experience. Interaction with the environment merely triggers pre-established programmes and stabilises the genetically specified synaptic organisation ("functional verification" hypothesis¹⁸). Dysfunction at critical periods of development may, however, lead to the regression of preformed, but already specified, synaptic connections¹⁸.

On the other hand, "empiricists" postulate that, to a large extent, the activity of the system specifies its connectivity, for instance by orienting the growth of the nerve terminals (see refs 17 and 19) or tracing pathways in more or less random networks^{20,21}. Third, as a compromise between these two attitudes, we have postulated^{22,23} ("selective stabilisation hypothesis"; see also refs 24 and 25) that the genetic program directs the proper interaction between main categories of neurones, for instance through the mechanisms presented below. However, during development within a given category, several contacts form at the same site; in other words, a significant but limited "redundancy" or fluctuation of the connectivity exists. The early activity of the circuits, spontaneous (in the embryo) and evoked (after birth) would increase the specificity or the order of the system, by reducing this transient redundancy. As a mechanism it was further postulated that the first synaptic contacts to form may exist under, at least, three states (synaptic plasticity): labile (L), stable (S) and regressed (D), the growth process being viewed as the emergence of labile states (Not→L). The labile and stable states would transmit nerve impulses but the regressed one (obviously) would not.

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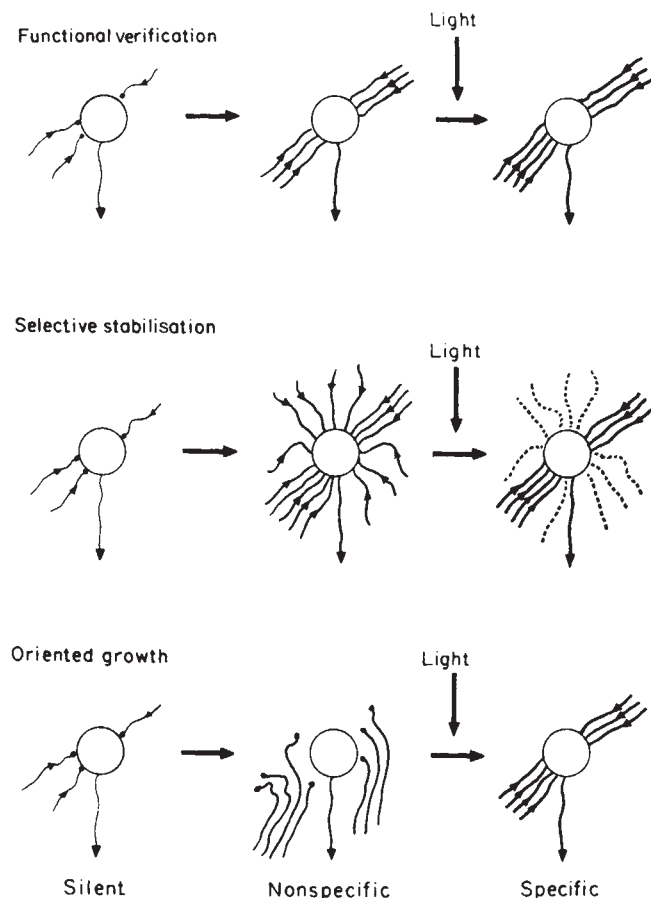


Fig. 1 Hypotheses regarding the effect of functional activity on the specification of a neuronal network: here a cell from the visual cortex which, in the adult, is specific for "orientation"¹¹. According to Buisseret and Imbert²⁰, in the course of development the first active units to appear are "nonspecific" and respond to stimuli moving in any direction of the visual field. Subsequently, they give rise to "specific" units activated by light spots moving along only one precise orientation. The three possibilities considered deal with changes in the connectivity but alternative (or additional) mechanisms may take place such as changes of efficacy of excitatory and/or inhibitory synapses, growth of new sets of connections and so on.

The labile state may either become stabilised (L→S) or regress (L→D), regression being irreversible. (Regrowth (Not→L) may however take place.) An essential statement of the theory²² is that the transitions of the labile state of the synapse (L→S and L→D) are regulated, in an "epigenetic" manner, by the total activity of the postsynaptic cell, including the activity of the considered synapse. Accordingly, the precocious activity of the developing network would "stabilise selectively" particular synapses among the many equivalent contacts which emerge during growth and thereby create diversity and specificity (in a concomitant manner the non-stabilised contacts will regress). As a consequence, a critical period exists in the development of a nerve terminal when it requires a given pattern of activity to become stable.

This selective stabilisation hypothesis has been formalised and applied to a few systems²². One of its advantages is that it may afford an economy of genes. Indeed some of the genes which dictate, for instance, the general rules of growth, the stability properties of the immature synapses (postulate 1), the regulation of their stability by the activity of the immature synapse (postulate 2), the integrative properties of the postsynaptic neurone, may be shared by different categories of neurones or even be common to all neurones. The set of genes involved (the genetic envelope) should therefore be smaller than if each synapse were determined individually. The amplitude of the limited redundancy subjected to functional epigenesis may vary

from one area of the nervous system to another and with the "complexity" of the organism considered. Negligible in some invertebrates, it becomes most significant in high vertebrates.

In the following, we shall review experimental observations taken primarily from vertebrate neuromuscular junction and cerebellum which bring some support to the selective stabilisation hypothesis. Finally, we shall discuss biochemical mechanisms which may account for the stabilisation process and therefore make it more plausible.

Selectivity of surface recognition reconsidered

The exquisite specificity of synapse formation and its interpretation in terms of selective recognition between cell surfaces has often been emphasised¹¹⁻¹⁶ but functional synapses between non-homologous pairs of cells^{12,14,26-28} may form. In mammalian cerebellum the afferent mossy fibres normally contact granular cell dendrites. By various means²⁹⁻³⁸, the granular cells can be destroyed. In spite of the absence of their normal target, the mossy fibres make both anatomical^{29,38} and functional³³ synapses on another category of neurones: the spines of the Purkinje cell dendrites. Functional heterologous synapses have also been obtained in the frog between the vagus nerve and the skeletal sartorius muscle transplanted into the thoracic region^{39,40} and in the rabbit between the thoracic vagus and the diaphragm⁴¹; their occurrence has also been reported with the sympathetic ganglion after denervation⁴²⁻⁴⁵ or *in vitro*, in tissue culture^{46,47}, even between cells which under normal conditions do not synthesise complementary transmitter and receptor⁴⁸.

These few examples illustrate that, in the absence of the normal target or after deviation of the axons from their usual route, functional synapses may form between non-complementary partners as long as the receptor for the neurotransmitter is present in the postsynaptic cell. In these instances, at least, the selectivity of surface recognition does not appear as stringent as one would expect from the strict "chemo-affinity" hypothesis. Compatibility between cell surfaces rather than complementarity might suffice for a synapse to be formed and additional mechanisms have to be invoked.

Variations in adult connectivity

If the paradigm of a highly selective and genetically determined development of synaptic connections were absolutely correct, one would expect the anatomy of the adult nervous system to be identical in genetically identical individuals. To obviate the genetic heterogeneity of a natural population, organisms have been studied that reproduce parthenogenetically: a small crustacean (*Daphnia magna*)⁴⁹ or even a viviparous tropical fish (*Poecilia formosa*)⁵⁰. In a clone of such organisms, the different individuals have the same genetic equipment. The three-dimensional reconstruction of the fine structure of the optic system (eye and optic ganglia in *D. magna*) reveals that the gross features of the anatomy, in particular the number of cells, are invariant. A significant variability appears, however, in the branching and synaptic patterns of their processes at a resolution of a few tenths to a few micrometres. The variation seems to be as great when a given neurone is compared with the symmetrical cell on the opposite side of the same animal and with the same cell in other members of the clone. In spite of genetic homogeneity the "exact" form of identifiable neurones and of their processes shows a significant dispersion from one individual to another. The limits of genetic determination have been reached.

Activity of the growth cone and of developing nerve terminals

The primitive contacts present early in development transmit nerve impulses, long before the differentiated synapses of the adult are formed. In chick embryo, for example, spontaneous movements begin as early as 3 d of incubation⁵¹ and are of neurogenic origin⁵¹⁻⁵⁴. The rat foetus also moves spontaneously after 15 d of life⁵¹. At this precise date exploratory fibres enter the diaphragm and establish functional contacts (in 3 of 30

cells impaled) even before the presence of acetylcholinesterase can be detected by the Koelle method⁵⁵. One day later, however, it is claimed that a single localised spot of acetylcholinesterase is present in 30% of the myotubes and distributed at random on the surface of the myotubes (although there is no convincing demonstration of this last point). In the majority of the cells impaled evoked postsynaptic potentials are recorded. In chick slow muscle anterior latissimus dorsi (ALD) a similar event takes place except that several spots of cholinesterase are observed at regular intervals along the length of the developing muscle fibre and persist in the adult fibre⁵⁵. This post synaptic localisation process concerns both the esterase⁵⁶⁻⁵⁹ and the cholinergic receptor protein which, dispersed evenly on the surface of the developing myotube, soon become concentrated under the ingrowing nerve terminal⁶⁰⁻⁶². In tissue cultures the rudimentary synaptic contacts which form also transmit nerve impulses⁶³⁻⁶⁶. Even the growth cone or its filopodia show anatomical features indicating presynaptic or postsynaptic relationships with cell bodies or processes⁶⁷⁻⁷⁰ with local accumulation of vesicles or postsynaptic thickenings and may give rise to typical postsynaptic potentials⁷¹. Chemical transmission therefore exists days before the pre- and postsynaptic differentiations of the mature synapse become apparent.

Cinematographic films of neurones in tissue culture show that the growing processes^{72,73} are in perpetual motion. They advance, divide, retract, start again until a contact with a cell body becomes stable. It seems that through trial and error only a few of the transient contacts made by the growth cone or the developing neurites are selected. An interesting possibility is that such a behaviour accounts at least in part, for the fluctuation observed in the adult connectivity and that the activity of the growing processes play a role in this selection.

Spontaneous regressive phenomena

In the course of neurogenesis, regressive phenomena have been reported to occur at two distinct stages. (1) At the time of neuroblast proliferation or immediately after, a significant fraction of neuronal somas die⁷⁴⁻⁷⁸. For example, the ventral horns of the spinal cord of *Xenopus* tadpole may contain a maximum of 5,000 or 6,000 cells but 60 d later, at the time of metamorphosis, only 1,200, the adult number, persist. The number of cells which disappear during this period could even be larger (10,000) than the overall difference in total cell number⁷⁹. Similar events take place in the spinal cord of chick embryo⁷⁸ and foetal mouse (between days 10-15 of intra uterine life^{80,81}) and in several other parts of the nervous system (see ref. 77). At later stages of development a second regressive phase affects primarily axon collaterals and dendritic branches without significant changes in cell number. For example, in foetal rat diaphragm, at d 17, soon after the dense acetylcholinesterase (and acetylcholine receptor) deposit appears in the middle of the muscle fibre, motor axons develop multiple branches and make a complex network which increases in size until birth⁵⁵. Observation of the endplate by electron microscopy shows several synaptic profiles in contact with the same synaptic folds. The "complexity" of the endplate potential (its stepwise increase of amplitude as a function of stimulus strength⁸²) furthermore reveals that the several motor axons which converge on a given endplate are functional^{55,82-84}. During the first postnatal week, the number of steps of the complex endplate potential progressively decreases: one of the endings finally dominates the others. At 3-5 weeks, all the synapses are innervated by a single nerve terminal and non-innervated muscle fibres are not seen. More than 60% of the functional contacts have, therefore, disappeared. The fine structure of the endplate during the regression phase does not reveal any gross degeneration of axonal branches and the number of axons in the ventral roots does not change significantly⁸³. These observations have been repeated with other muscles⁸³, in particular with avian ALD⁵⁵ where each of the several endplates present per muscle fibre becomes innervated by several axon terminals.

Since the number of muscle fibres^{85,86} and of motor axons^{87,88,89} remains constant during this period, the number of muscle fibres innervated by a single motor neurone, that is the size of the "motor unit", should change^{88,89,89}. Indeed, during postnatal development the average size of the motor units decreases. In the 3-d-old rat soleus, a single motor unit tension is about 1/4 of the total muscle tension⁸³. In the adult, the fields of innervation of the motor neurones no longer overlap and a single motor fibre commands the contraction of a five times smaller set of fibres. This permits a progressive recruitment of a larger number of independent units and therefore a graded contraction of the muscle. The regressive process, which leads to the establishment of the "one motor terminal one muscle fibre" relationship and the corollary decrease in size of the motor units, corresponds to an improvement of the motor command of the muscle. One may say that the selective elimination of redundant synapses increases the "specificity" of muscle innervation.

In the adult cerebellum, the majority (more than 90%) of the Purkinje cells receive only one climbing fibre^{17,90}. Unit recordings in 8-9-d-old rats show that, at this early stage, the response of the Purkinje cell to climbing fibre stimulation is "complex" as in the case of the immature neuromuscular junction: more than 50% of the Purkinje cells would be innervated by at least two distinct climbing fibres. A subsequent regressive phenomenon leads, as nearly as 15 d after birth, to the one-to-one relationship of the adult^{91,92}.

In these instances, the genetic program of the organism allows for an overproduction of labile and functional contacts but during maturation a significant fraction of them subsequently regresses.

Effects of target cell and its functional activity on synapse stabilisation

In the chick embryo⁹³⁻⁹⁵ and in amphibians⁹⁶⁻⁹⁸ early limb bud extirpation causes a massive depletion of both motor neurones and sensory ganglion cells accompanied by a decrease of the enzyme choline acetyltransferase in the spinal cord⁹⁹. Removal of the peripheral target cells dramatically enhances the "normally occurring cell death". An interaction with the periphery is therefore required for the neurone to continue its development.

In the mutant mouse "staggerer"³¹, similarly, a massive regression of the parallel fibres and granular cells occurs at late stages of development¹⁰⁰ and has been attributed to the failure of parallel fibres to establish normal synapses with the Purkinje cells (which show electrophysiological signs of abnormality¹⁰¹ and lack a characteristic membrane protein¹⁰²).

In none of these instances, however, has evidence been presented that the actual activity of the postsynaptic cell has any effect on these regressive phenomena. To test this possibility, several cholinergic agents or toxins known to block selectively neuromuscular transmission were injected into developing chick embryos¹⁰³⁻¹¹². These drugs stop the spontaneous movements of the embryo which may, nevertheless, survive until hatching. Such treatment causes a marked atrophy of skeletal muscles which show signs of delayed differentiation and of regression¹⁰³⁻¹¹². Pre- and postsynaptic toxins have similar effects, which suggests that the actual "functioning" of the muscle by way of the synapse is necessary for its growth, histogenesis and maintenance. Interestingly, these compounds affect the innervation¹⁰⁷⁻¹⁰⁹. For example, a typical postsynaptic blocking agent, the α -toxin from the venom of *Naja nigricollis*, causes the almost complete absence of typical motor endplates as revealed by the Koelle reaction (the embryos were injected at 3, 8 and 12 d of incubation with high doses of α -toxin and observed at 16 d). The same happens with another postsynaptic antagonist: *d*-tubocurarine¹¹⁰⁻¹¹². In addition, and in a rather unexpected manner, despite the fact that α -toxin binds selectively to the nicotinic receptor site, marked regression takes place on the presynaptic side of the junction, as manifested by

a decrease in the specific and total activity of choline acetyltransferase in the muscle and sciatic nerve¹⁰⁷ and a reduction (more than 50%) in the total number (myelinated and non-myelinated) of axons in the ventral root of the spinal nerves (in the dorsal roots no significant change takes place)¹⁰⁹. A selective loss of the motor neurones takes place. This regression seems to be less significant with the presynaptic botulinum toxin at the concentration tested possibly because of an intrinsic effect of the toxin on the nerve terminal (for example it is known that botulinum toxin poisoning causes axonal sprouting at the adult neuromuscular junction¹¹³.) Although a direct action of the α -toxin on the neurones of the spinal cord^{114–115} cannot be ruled out in these experiments, no α -³H-toxin binding was detected in spinal cord extracts; in addition, the number of fibres counted in the spinal roots clearly shows that the effect of the α -toxin is limited to the cholinergic motor neurones¹⁰⁹.

How can an essentially postsynaptic block influence, then, the motor nerve terminal? The possibility that the α -toxin interferes with the "recognition" step at the early stages of synapse formation is made unlikely by the observation that primitive junctions (as well as the content of choline acetyltransferase) in embryos injected at the 4th d of incubation do not significantly differ from those of the non-injected control until the 12th d of incubation. Similarly, *d*-tubocurarine or α -bungarotoxin do not prevent synapse formation *in vitro*^{116–119} or re-innervation of adult rat diaphragm¹²⁰.

In agreement with this interpretation are recent findings on the effect of another snake α -toxin (from *Bungarus multicinctus*) on the development of retinotectal synapses in the toad: *Bufo marinus*¹²¹. The α -toxin was applied to a circumscribed region of tectal surface where it blocks the postsynaptic acetylcholine receptor in a stable manner for several weeks. In regeneration experiments, the optic nerve initially invades the toxin treated regions and subsequently retracts. In situations where the optic nerve was intact, chronic application of α -toxin results in a subsequent loss of optic nerve synaptic terminals. As in the case of the neuromuscular junction the stabilisation of developing nerve terminals requires the functional interaction of the neurotransmitter with its postsynaptic receptor.

Preliminary evidence suggests that the late phase of synapse maturation, that is the regression of multiple innervation, is also coupled with activity. Section of one of the tendons of sartorius muscle in infant rats indeed significantly delays this selective regression⁸⁴ but it is not yet known whether neonatal tenotomy modifies the chronic firing of the motor neurones. Also, in the cerebellum, as a consequence of the absence of the Purkinje cell major input (the lack of granular cells and their parallel fibres in X-irradiated rats^{122–123} and "weaver"¹²⁴ mouse), the multiple innervation by climbing fibres observed in the infant rats persists in adulthood.

Biochemical models for selective stabilisation

These few biological examples briefly reviewed provide some support for the "selective stabilisation" hypothesis. In particular, it is clear that (1) at critical stages of development neuronal bodies as well as growing neurites may exist under a functional labile state susceptible to death or regression (postulate 1); (2)

the number of neuroblasts and nerve connections produced at critical stages of development is significantly larger than that persisting in the adult (postulate 2); (3) the activity of the network may contribute in a direct or indirect manner, to this stabilisation process (postulate 3): in particular, retrograde signals emitted by the postsynaptic soma seem to have a significant role. Regarding this last point, the role of the "nerve growth factor" as a retrograde signal in the development of the sympathetic ganglion has already been extensively documented and reviewed^{125–127}.

The following discussion deals with biochemical processes, relevant to the mechanism of synapse stabilisation. We shall distinguish events taking place on the postsynaptic side of the synapse (primarily the "localisation" of the receptor protein) from those concerning the nerve terminal and its selective "maintenance" or stabilisation. The examples and reasonings presented concern the developing neuromuscular junction in vertebrates but might be adapted to more complex neuronal networks.

Postsynaptic 'localisation' of the receptor protein

This discussion deals with the 'localisation' of the acetylcholine receptor, but it should be borne in mind that localisation of acetylcholinesterase takes place simultaneously. In the adult neuromuscular junction the density of acetylcholine receptor sites counted with radioactive snake α -toxins is 100–1000 times higher than in extrasynaptic areas^{128–134}. It reaches an average of 8,000–9,000 sites per μm^2 (refs 128–132) in mouse junction with a higher density in the top ($30,000 \pm 60,000$ sites per μm^2) than in the bottom of the synaptic folds^{133,134} and up to $50,000 \pm 15,000$ sites μm^2 in *Electrophorus* electroplaque (refs 135–137 and J. P. Bourgeois, J. L. Popot, A. Ryter and J. P. C., unpublished). The sub-synaptic membrane consists of a closely packed (lattice) assembly of the receptor molecules. Once formed the subsynaptic membrane appears remarkably stable: after denervation, the main features of the endplate region^{138–140}, the dense deposit of acetylcholinesterase revealed by the Koelle technique^{2,141} and the high sensitivity to iontophoretically applied acetylcholine^{142–144} persists for weeks. In *Electrophorus* electroplaque, the density of α -³H-toxin sites in the subsynaptic membrane decreases by less than 50%, 52 days after denervation (refs 136, 137 and J. P. Bourgeois, J. L. Popot, A. Ryter and J. P. C., unpublished). No significant tendency for lateral diffusion of the receptor protein exists either in the adult "uncovered" neuromuscular endplate^{145,146}. In the developing rat or chick myotubes, the density of acetylcholine receptor sites lies in between that of the extra and subsynaptic areas in the adult synapse ($1,500/2,000$ α -toxin sites per μm^2)^{147–149}. Although the actual translation mobility or the receptor protein in embryonic cells has not been measured yet, it should be close to that of the bulk proteins in a highly fluid cytoplasmic membrane^{150,151} that is much higher than in the subsynaptic membrane of the adult synapse.

The turnover rate of the cholinergic receptor protein in junctional and extra-junctional areas has been measured either with α -¹²⁵I-bungarotoxin (the particularly high stability of the toxin-receptor complex makes possible measurements of turnover rates faster than its own half life (9–15 d)^{148,152–154}) or directly after pulse labelling of the receptor protein by ³⁵S-methionine^{155,156} or heavy isotopes¹⁵⁷ followed by immunoprecipitation and/or purification. In developing calf myotubes *in vitro* the two methods give identical turnover rates¹⁵⁸. Table 1 shows that the degradation of the receptor protein occurs at a rate considerably faster in extra than in subsynaptic areas both *in vivo* and *in vitro* and the rate in the extrasynaptic areas of the adult muscle after denervation is close to that observed on the surface of the developing myotube. The degradation process is blocked by inhibition of oxidative phosphorylations: DNP, cyanide (possibly because of an energy-dependent "internalisation" process) but insensitive to inhibitors of protein synthesis (cycloheximide, puromycin)¹⁴⁸. The rate constant of

Table 1 Degradation of the cholinergic receptor protein

Extrasynaptic: developing myotubes <i>in vitro</i> :	
—decay of bound α -toxin	16–28 h (ref. 148)
—chase of ³⁵ S labelled receptor and decay of	16 h (ref. 158)
bound α -toxin	
adult rat diaphragm:	
<i>in vivo</i>	19 h (ref. 153)
<i>in vitro</i>	8–11 h (ref. 154)
Subsynaptic:	
adult rat diaphragm:	
<i>in vivo</i>	7–5 d or more (ref. 153)
<i>in vitro</i>	6 d or more (ref. 154)

degradation of the extrasynaptic receptor appears independent of the culture conditions and in particular does not vary with the growth rate of the developing myotubes (ref. 149 and J. Merlie, unpublished). Under these conditions the regulation of the number of receptor molecules therefore depends directly on its rate of synthesis.

Pharmacological differences have been reported *in vivo* between the subsynaptic and the extrasynaptic receptors^{60,61}; in addition, noise measurements reveal that the opening time of the cholinergic ionophore increases after denervation⁴; yet, they may not reveal differences in the primary structure of the two classes of receptor molecules but, for instance, differences in packing or environment of the receptor protein in the membrane. In agreement with this interpretation are the following observations. (1) *In vitro* the binding constants for agonists and antagonists of the membrane-bound receptor do not change in chick embryo leg muscles from 8 d (extrasynaptic) before hatching until 70 d after hatching (subsynaptic) (G. Giacobini, unpublished); the receptor proteins purified by affinity chromatography from extra and subsynaptic regions of rat diaphragm have been reported to show identical binding properties in one laboratory¹⁶¹ but different in another one¹⁶¹. (2) The equivalence point by immunoprecipitation seems to be identical for the two receptors (ref. 162, and H. Sugiyama, unpublished). (3) These two molecules have the same hydrodynamic properties¹⁶². Only isoelectric focusing unambiguously separates the two receptors, revealing a charge difference¹⁶² but interconversion between the two structural forms has been obtained *in vitro*¹⁶³. The most likely interpretation of the data is that the extrasynaptic and subsynaptic receptors derive from the same protein molecule, the observed difference would result, for instance, from a covalent modification¹⁶⁴ but a different subunit composition of the two forms is not excluded.

Little is known yet about the factors which regulate the distribution and concentration of acetylcholine receptor in developing neuromuscular junction; however, the increase of sensitivity in extra-junctional areas after denervation is accounted for entirely by a neo-synthesis of receptor molecules^{155,165,166}. Direct electrical stimulation of adult denervated muscle through chronically implanted electrodes (refs 167–169 and T. Lømo, unpublished) or of embryonic non-innervated myotubes¹⁷⁰ maintained *in vitro* abolishes the hypersensitivity to ACh in the extra-junctional areas. The result depends critically on the amount and pattern of the stimuli. Maximal rate of decline of ACh sensitivity is approximately exponential with a half time in the range of that reported for the degradation in extra-synaptic areas^{168,169}. It is likely that optimal stimulation blocks receptor synthesis, the acetylcholine sensitivity falling at a rate

determined by the degradation of the extra-junctional receptor¹⁶⁹ (recent experiments¹⁷⁰ actually show that activity slows down this rate by about a factor of two).

To account for the localisation of the receptor protein and for eventual intercorrelations between developing synapses mediated by the postsynaptic cell we propose, that during development (Fig. 2):

(1) The receptor may exist under two interconvertible forms: *l*, labile and diffusible and *s*, stable, resistant to degradation and immobilised.

(2) The *s* state derives from the *l* state by way of a stabilisation reaction $l \rightarrow s$ which is not reversible. This reaction takes place when both an anterograde factor liberated by the nerve terminal during activity and an "internal coupling factor" are present above a critical concentration and within a given lapse of time, on the two faces of the subsynaptic membrane.

(3) On the membrane area underlying the developing nerve terminals, the neurotransmitter, in addition to its electrogenic effects, triggers the emission of a signal whose amplitude is given by the concentration of internal coupling factor which propagates inside the cell. (The propagation of the internal signal may result from a regenerative process. The initiation of the signal may take place with a significant delay in such a manner that its amplitude would reach the threshold for stabilisation only at a certain distance from the synapse. This would prevent, or delay, the self-stabilisation of the emitting synapse.)

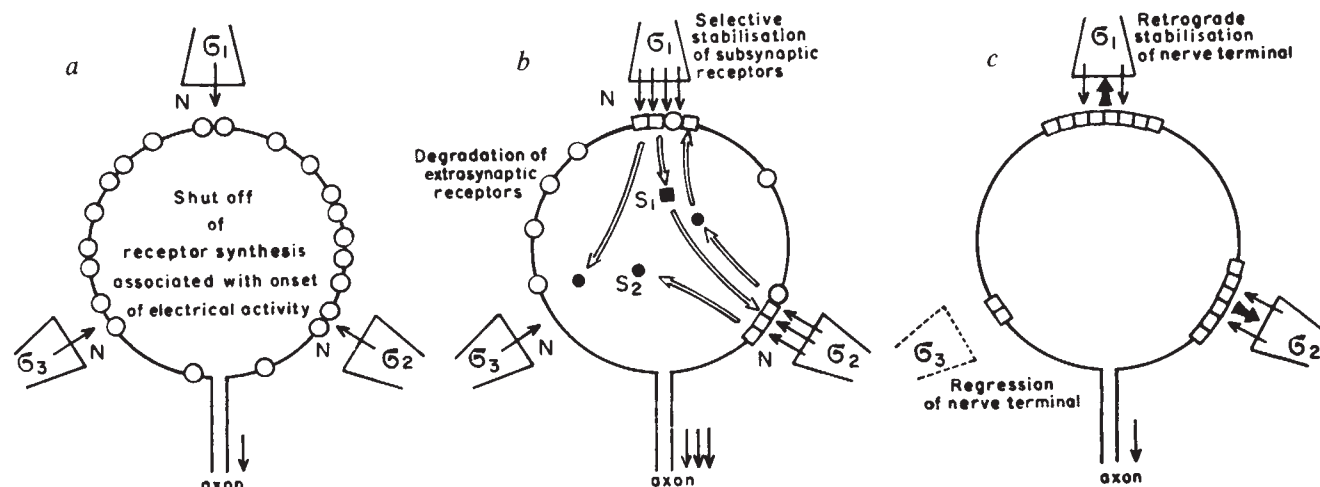
(4) The synthesis of the labile form of the receptor protein stops in the postsynaptic cell when activity starts in the myotube. An internal shut-off factor would act selectively on the protein synthesis machinery.

Accordingly, the selective localisation process would correspond to a management of a fixed and limited stock of *l* via lateral diffusion and stabilisation reactions.

This model is being formalised¹⁷² and may account for the redistribution of the receptor protein during localisation. The still hypothetical stabilisation reactions of the receptor molecule might for instance be a covalent modification of the receptor molecule such as a phosphorylation^{173–175}, an adenylation^{175,176}, a glycosylation^{177,178} or any other mechanism providing protection against proteolysis^{179,180}. The modification might for instance change the tendency of the receptor molecule to make aggregates under which it would be altogether resistant to degradation and immobile. It might also facilitate the anchoring of the receptor molecule inside the postsynaptic cell to fibrillar proteins¹⁸¹: actin, tubulin, or collagen, or, on its external surface to the basement membrane or any "cleft substance".

The most likely candidate for the "anterograde factor" is the neurotransmitter^{104,107,111,164}. This role in localisation has been

Fig. 2 The selective localisation of the receptor on the surface of a neurone receiving several nerve terminals. The labile and mobile state of the receptor for the neurotransmitter is represented by a circle, the stable and immobilised one by a square. The different signals postulated are represented by arrows: N, the anterograde factor, for instance the neurotransmitter; S, the internal coupling factor; a thick arrow indicates the retrograde factor. At the onset of activity a "shut off" factor stops the synthesis of labile receptor molecules. The model allows for the segregation of different receptor molecules if the nerve terminals secrete different anterograde factors.



challenged¹¹⁸ despite the fact that it provides a simple explanation for the segregation of different receptor molecules in a complex situation such as that of a neuronal soma receiving nerve terminals with different neurotransmitters. Other factors liberated by the nerve terminals such as, for instance, ATP^{182,183} or some of its degradation product enzymes or polypeptide hormones might have to be considered. The liberated ATP might offer a convenient energy supply for reactions taking place on the external surface of the membrane. Alternative (or additional) processes which do not require the emission of a diffusible signal might also have to be envisioned such as a "patching"^{61,181} of the receptor molecules in the postsynaptic membrane; spontaneous or resulting from a direct physical contact with the nerve terminal.

The postulation of one (or several) internal "coupling factor(s)", in addition to the membrane potential¹⁸⁴ is not necessary to account for the localisation of the receptor protein in the focally innervated muscle fibre. It may, however, offer plausible explanations for the development of complex patterns of synapses on neuronal somas (Fig. 2) or dendrites or, in the case of the avian ALD, of a regular distance between synapses. In any case it allows for interactions between synapses on a given soma in a more discriminative manner than the membrane potential. The postulated mechanism for the propagation of the signal is even more hypothetical than its very existence but plausible reactions may be written down which lead to the propagation of a "chemical" wave. (They might for instance be those of the nucleotide cyclase protein kinase and phosphatase system if, in one manner or another, the protein kinase target of the cyclic nucleotide exerts a positive feedback on the nucleotide cyclase.)

The "shut-off" of protein synthesis may be triggered by the internal coupling factor or by a different one. Possible candidates are the often evoked Ca^{2+} ions^{185,186}, cyclic nucleotides^{173,187} or both. Ca^{2+} would be a rather simple "shut-off" factor since it might enter through the cholinergic ionophore¹⁸⁸⁻¹⁹¹ or the tetrodotoxin sensitive ionic channels¹⁹²⁻¹⁹⁴ when they are activated. Signals directly coupled with the mechanical contraction of the muscle may also have a role.

In any case, despite its non-uniqueness the proposed model gives rise to several predictions which may offer experimental tests for the validity of its critical assumptions.

(1) If localisation corresponds to the management of a limited pool of receptor protein, changing the size of this pool by selectively blocking receptor synthesis before the arrival of the nerve terminals should interfere with localisation and/or reduce the number of endplates formed in a muscle like ALD. The "immunity" to innervation of the extrasynaptic areas in an adult muscle fibre would then simply result from the absence of receptor molecules available in the extrasynaptic membrane to form a novel endplate.

(2) The activity of the postsynaptic cell is expected to regulate the synthesis of receptor through the shut off factor but also the management of the membrane pool of receptor through the stabilisation reaction. Once the synthesis of receptor has stopped, changes in the program of activity of the developing nerve terminals by chronic stimulation (or blocking) may lead to the formation of supernumerary endplates (or prevent localisation) or, thanks to the internal coupling factor, for instance modify the distance between endplates. In avian ALD this may occur¹¹⁰⁻¹¹². In agreement with these views is the recent¹⁹⁵ observation that the spontaneous activity markedly differs in the slow ALD with multiple endplates and the focally innervated fast PLD from chick embryo (16-18-d-old): ALD fires continuously at a rate of 0.2 to 5 Hz, while in PLD only occasional activity is seen at a rate of 4 to 8 Hz. Chronic blocking of synaptic transmission may also modify the pattern of endplates and their relative distance in these muscles¹¹⁰⁻¹¹².

(3) According to the theory, the development of a characteristic geometric pattern of synapses may be determined by the exact temporal pattern of activity integrated by the postsynaptic cell. To illustrate this point, if Ca^{2+} is the shut off factor its

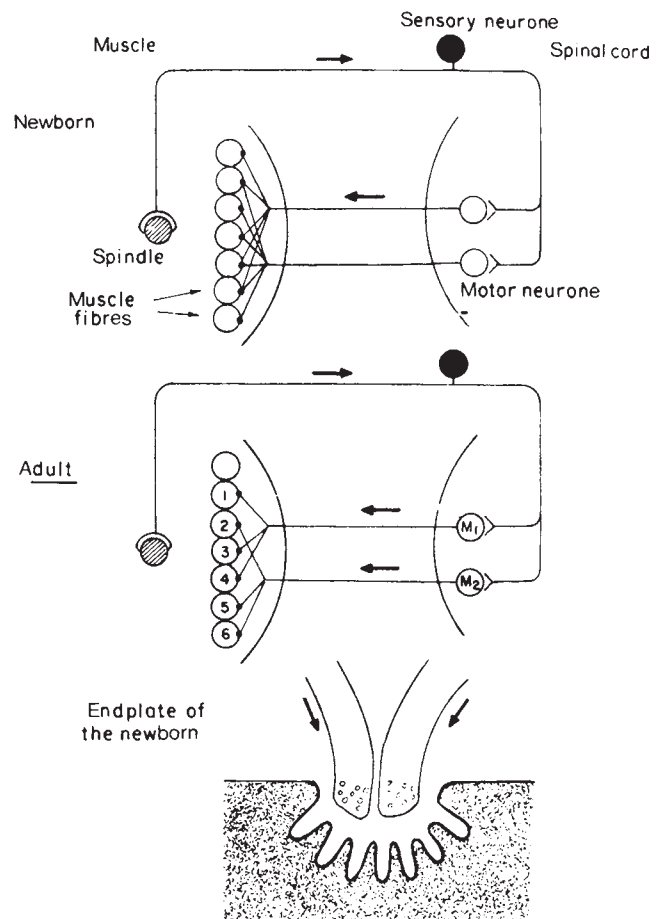
internal concentration might be regulated by two competing kinetic processes: the opening of the ionophores in the post-synaptic cell and the pumping rate of calcium by the mitochondria. A critical firing rate shall then be required to let the internal Ca^{2+} concentration reach a critical value to turn off the synthesis of receptor.

Presynaptic selection of a single nerve terminal at multiply-innervated endplates

Less information is available as yet on this second step of synapse maturation (Fig. 3), we may nevertheless propose that: (1) all the motor nerve terminals converging on a given endplate receive the same average number of impulses but in a randomly non-synchronous manner, (2) at the peak of the multi-innervation stage, all terminals share almost equivalent areas of the stabilised subsynaptic membrane surface and/or dispose of a limited stock of a postsynaptic "retrograde factor" x , (3) the arrival of impulses cause an increase of surface occupancy and/or of utilisation of x , (4) when the surface occupied by the nerve terminal (and/or the amount of x utilised) becomes lower than a critical value this nerve terminal regresses.

This set of minimal assumptions may explain both the stabilisation of a single nerve terminal and the constant size of the motor units. Accordingly, the size of the motor unit should be determined by the ratio of the number of muscle fibres to that of motor neurones. Indeed, it is known from human patients that a partial neurogenic atrophy (which reduces the number of motor neurones) causes a decrease in the number

Fig. 3 The differentiation of motor units in a skeletal fast muscle by retrograde selective stabilisation of nerve terminals. In the neonate one motor neurone innervates many more muscle fibres than in the adult and each muscle fibre receives several nerve terminals. A few weeks after birth only one nerve terminal persists per muscle, the others regress.



of motor units with a marked increase in their size¹⁹⁶⁻¹⁹⁸. An increase of surface occupancy by nerve terminals as a consequence of activity has been described^{199, 200}, but the biochemical mechanisms by which a nerve terminal becomes stabilised once a critical surface of the postsynaptic membrane is occupied remains obscure. The postulated retrograde factor (substance *x*) has not been identified chemically; in the case of the neuromuscular junction: the concentration of calcium in the cleft¹⁰⁸ or of any nerve growth factor are possible candidates. An interesting possibility would be the direct covalent modification of the fibrillar or tubular proteins engaged in the maintenance of the shape of the nerve terminal and thereby regulating its internal "cytoplasmic flow".

Conclusions

From a biochemical point of view, the selective stabilisation hypothesis appears plausible. Four different signalling mechanisms have been postulated to govern the evolution of a developing synapse: (1) for the postsynaptic localisation of the receptor protein: an anterograde factor liberated by the nerve terminals (possibly the transmitter) which governs the stabilisation reaction of the receptor, an internal shut off signal which informs the protein biosynthesis machinery (transcription and/or translation) of the activity of the postsynaptic cell, an internal coupling factor which permits exchange of information between synapses and 2) for the selective presynaptic stabilisation of the nerve terminals, a retrograde factor emitted by the postsynaptic cell. Additional mechanisms and regulatory signals may have to be postulated in the future to account for the complete description of the stabilisation and maturation of the synapse which is certainly a complex process lasting for days or even weeks. In any case, at this level of understanding the word "trophic factor" is of no help and should be abandoned.

The basic assumption of the theory that an increase of "specificity" accompanies the active stabilisation of particular synapses (and the regression of others) remains to be tested with neuronal networks (cerebellum, visual cortex) more complex than the motor innervation of the striated muscle. For instance, an intriguing possibility would be that in the much studied visual cortex (Fig. 1) the acquisition of a given specificity such as the specificity of orientation¹⁸, results from such a selective stabilisation (Fig. 1). It would be of primary interest to investigate through intracellular recordings if the transition from "nonspecific units"²⁰¹ to "specific" ones would correspond to a decrease of synapse redundancy via the regression of transiently multi-innervated neuronal somas and to what extent the activity of the system, spontaneous and/or evoked, governs these transitions.

In a more general manner, these speculations place emphasis on critical features of developing neuronal networks which are directly accessible to the experiment: (1) their early activity (spontaneous or evoked) preceding synapse maturation: (2) the existence and limits of a transient redundancy (in the case of motor innervation the limits of the fluctuation are the boundaries of the muscle²⁰²); (3) an eventual modification of this redundancy by chronic changes of the early activity.

Even if the limits of the redundancy are narrow, they may be sufficient to provide an opportunity to save genes during the differentiation of neuronal networks and to provide a plausible biochemical mechanism for "learning" without postulating any synthesis of new molecular species.

Many theoretical aspects of this paper result from work done in close collaboration with Philippe Courrège.

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articles

Ophiolites in south-western Newfoundland

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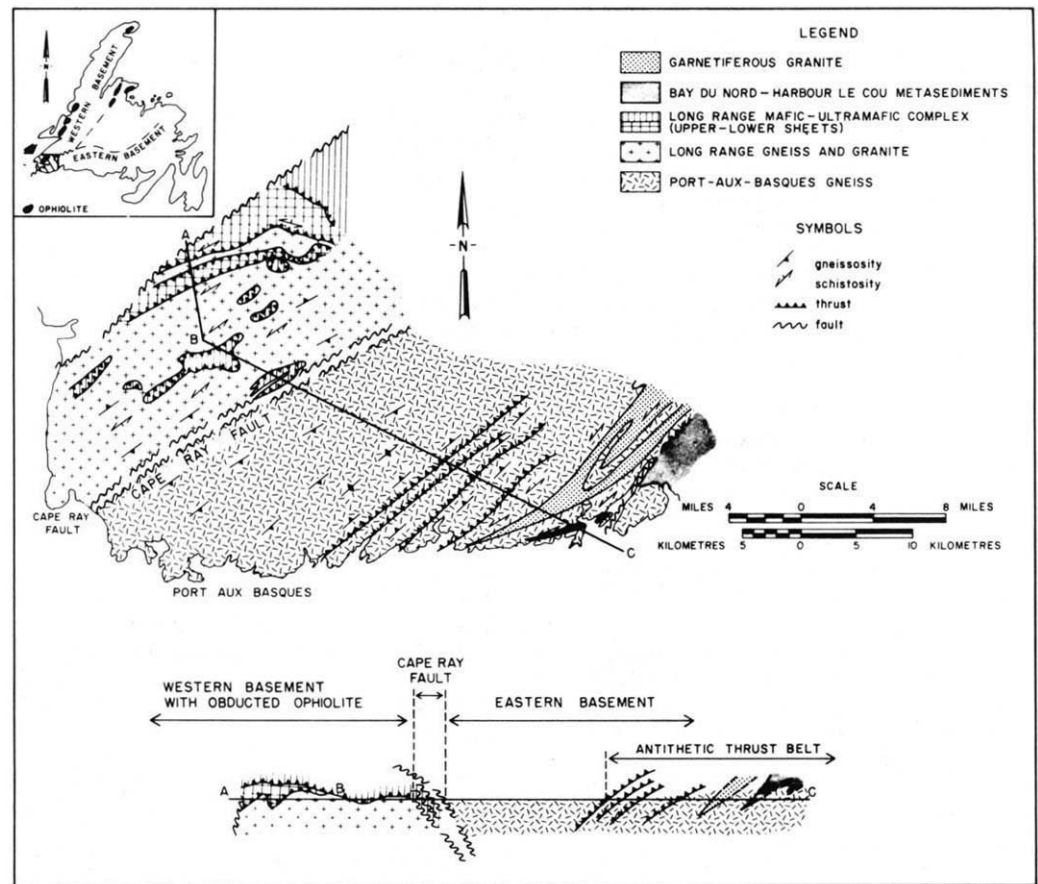
Ophiolite and ophiolite-related rocks have long been recognised in northern and western Newfoundland^{1,2}. The presence of these rocks has, to a great extent, controlled the plate tectonic models developed for the tectonic evolution of the Newfoundland Appalachians. Such models have been extrapolated through south and south central Newfoundland with little geologic control. I describe here the ophiolites in south-western Newfoundland, discuss their relationship to a cryptic suture, the Cape Ray Fault, and evolve an independent model, which I compare with the model developed for northern and western Newfoundland.

SOUTH-WESTERN Newfoundland consists essentially of two basement complexes separated by a zone of intense mylonitisation, the Cape Ray Fault. The Long Range Gneiss, to the North of the fault, forms part of the Western Crystalline Belt³ and, as such, defines the western margin of the Proto-Atlantic Ocean. The Port aux Basques Gneiss, to the South of the fault, forms part of the Eastern Crystalline Belt³ and defines the eastern margin of the Proto-Atlantic Ocean. The Cape Ray Fault has been interpreted as a cryptic suture, along which complete closure of the Proto-Atlantic Ocean took place⁴.

Lower sheet

Recent work (P. A. Brown, unpublished) in south-western Newfoundland has shown that the Long Range Mafic-Ultramafic Complex⁵ occurs as thrust sheets overlying a

Fig. 1 General geology of southwestern Newfoundland showing the relationship of the obducted ophiolite to the Cape Ray Suture and antithetic thrust belt.



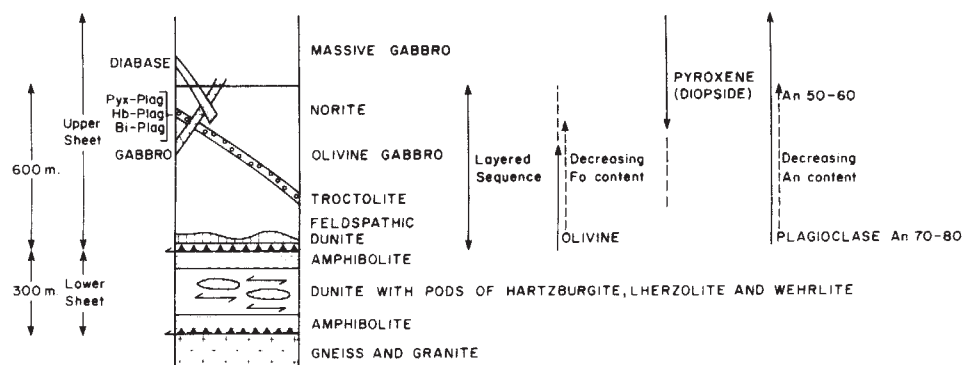
granitic gneiss terrane, the Long Range Gneiss (Fig. 1). There are two main thrust sheets, a lower, intensely tectonised sheet, and an upper, well preserved, less tectonised sheet (Fig. 2). The lower, dominantly ultramafic, thrust sheet consists of variably tectonised dunite, harzburgite, lherzolite and wehrlite. The harzburgites, lherzolites and wehrlites occur as tectonic 'pods' within highly serpentinised dunite. Original igneous textures are not preserved. There is an increase in deformation towards both the upper and lower contact of the sheet. The lower contact is defined by a highly schistose cumingtonite-pargasite-pyroxene schist. This schist is in tectonic contact with granite and granite gneiss of the basement and is interpreted as the basal thrust of the mafic-ultramafic complex. Locally this contact is marked by a serpentinite mélange, with blocks of serpentinite up to 50 cm in diameter set in a carbonate matrix. The upper contact is defined by a well-developed actinolite-zoisite schist which grades upwards into a locally thick sequence of well banded amphibolites. The banding is discontinuous and irregular and is igneous in origin. The amphibole characteristically shows a blue-

light green pleochroic scheme and is probably pargasitic in composition.

Upper sheet

The upper sheet, in contrast, is well preserved, little tectonised, and a lithological sequence can be outlined. Within the sheet there is a decrease in tectonism away from the Cape Ray Fault. The rock types vary from feldspathic dunite at the base, through a well layered sequence of troctolite, olivine gabbro, norite and anorthosite, and finally into massive, coarse to fine grained, gabbro at the top (Fig. 2). Two types of layering are present. A rhythmic layering of width 5–20 cm with olivine- or pyroxene-rich bases and plagioclase-rich tops. These show graded (mineralogical) bedding and slump features. The succession is nowhere observed to be inverted. At the base of the succession, olivine is the dominant mafic mineral. The percentage of olivine decreases upwards, and towards the top of the succession pyroxene becomes the dominant mafic mineral (Fig. 2). The second type of layering is cryptic and occurs on both a small and large scale. On a small scale, the composi-

Fig. 2 Generalised section of the Long Range Mafic-Ultramafic Complex.



tion of plagioclase shows a decreasing Anorthite content from the intercumulus crystals at the base to the cumulus crystals at the top of each band. On a large scale the plagioclase shows an overall change in composition from An_{70-80} at the base of the succession to An_{50-60} at the top. Olivine varies from $For_{90}Fa_{10}$ (cumulus) at the base to $For_{80}Fa_{20}$ (intercumulus) towards the top. The pyroxene appears to become more diopsidic towards the top. Dark green spinel (hercynite?) is found throughout the sequence.

Boundary regions

The boundary between the layered sequence and the massive gabbros is often marked by plagioclase–pyroxene pegmatites. The gabbro becomes finer grained upwards and is intruded by stringers and pods of a plagioclase–rich, potassium-feldspar-poor biotite granite. The entire sequence is cross cut by several types and ages of dykes. Pegmatitic pyroxene–plagioclase, hornblende–plagioclase, and biotite–plagioclase dykes cut the layered sequence. These are cross cut by gabbro dykes, which in turn are cross cut by diabase dykes with chilled margins.

The complex was emplaced along a series of thrusts that define a schuppen-type structure. There are two major thrusts. The lower (basal) thrust separates the lower sheet from the underlying granite and gneiss and most of the initial movement has taken place along this thrust. The upper (intraformational) thrust separates the lower from the upper sheet and results in a structural stacking of the mafic–ultramafic complex.

The Cape Ray Fault

To the south-east, the complex is bounded by a south-eastwards dipping mylonite zone, the Cape Ray Fault. The upper sheet becomes progressively tectonised towards this mylonite zone and, within it, is altered to a chlorite-actinolite schist. Most of the mylonite is derived from the granitic-gneissic basements that define the eastern and western margins of the Proto-Atlantic Ocean. This led to the Cape Ray Fault being interpreted as a cryptic suture along which complete closure of the Proto-Atlantic Ocean took place⁴.

In northern and western Newfoundland, the mafic–ultramafic rocks preserved as slices thrust onto the western crystalline belt (White Hills Peridotite and Bay of Islands Complex) have been shown to be ophiolite in origin^{1,6,7}. It is suggested that the similar setting (Fig. 1) of the Long Range Mafic-Ultramafic Complex indicates that this body is also of ophiolitic origin. This suggestion is supported by the similarity of the stratigraphy of the complex to that of the critical zone and upwards of many recognised ophiolites⁸ and specifically to that of the Bay of Islands Complex^{2,7}. Furthermore, the mode of emplacement, that is, schuppen type structures, is compatible with subduction–obduction tectonics. The presence of the complex only to the North-west and partially within the southwards-dipping Cape Ray Suture strengthens this interpretation and also indicates that the subduction zone must have dipped, at least in the final stages, to the Southeast.

The formation of the Cape Ray Suture implies an inter-continental continent–continent collision in south-western Newfoundland. It is likely that such collisions develop an antithetic thrust belt on the down dip side of the subduction zone⁹. Such a belt, involving high grade, shear zones, dipping to the north-west, and recumbent folds incorporating both basement and cover rocks, is found 20 km–30 km to the south-east of the Cape Ray Fault. The sense of movement is overthrusting to the south-east. Involved in this thrusting are garnetiferous leucocratic granites (products of extreme differentiation of calc-alkalic magmas¹⁰), which may be related to southeastwards subduction under the eastern crystalline belt.

The model proposed for the geological development of

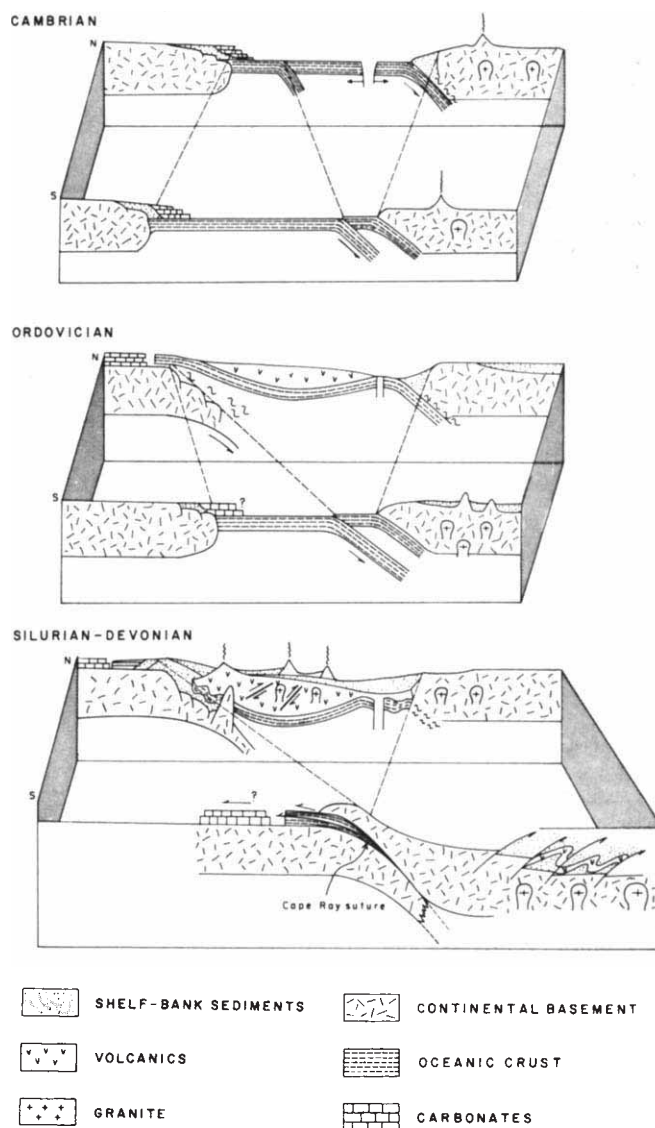


Fig. 3 Model for the closure of the Proto-Atlantic Ocean in Newfoundland. Northern sections after ref. 17.

this area is therefore:

(1) Southeastwards subduction under the eastern margin of the Proto-Atlantic resulting in calc-alkalic magmas and related intrusive differentiates.

(2) Closure of the ocean along the trace of the Cape Ray Fault with accompanying obduction of ophiolite onto the western margin and the development of an antithetic thrust belt.

(3) Final overriding of the western margin by the eastern margin resulting in internal thrusting and structural stacking of the ophiolite. The cessation of thrusting is probably related to the isostatic upward pressure of the downthrust western continental slab.

Although this model is, in general, similar to recent models developed in northern Newfoundland, there are significant differences: first, the obducted ophiolites to the north and west occur as large slabs thrust onto shelf-bank sediments and these sediments are themselves involved in the thrusting. In south-western Newfoundland the ophiolite is dismembered and is thrust directly on to a gneiss and granite basement. Second, the timing of final emplacement in the north is Middle Ordovician^{12,13}. In the Southwest the obduction event cannot, at present, be dated, but the related antithetic thrust belt affects cover rocks (the Bay du Nord Group) which contains Lower Devonian fossils¹⁴.

The different style of ophiolite emplacement in northern and southern Newfoundland suggests either that the Cape Ray Fault is a deep level exposure of the obduction event in the North (see Fig. 4, ref. 15) or that there are two independent styles of obduction. The first possibility is attractive in that it explains the lack of shelf-bank sediments and the dismemberment of the ophiolite in the southwest. It fails, however, to explain the development of an antithetic thrust belt of Acadian age if complete closure took place in the Middle Ordovician. Furthermore, the calc-alkalic garnetiferous granite involved in the antithetic thrusting has been dated at 415 ± 15 Myr (R. Cormier, personal communication), indicating that subduction continued under the eastern continent in post Middle Ordovician times.

The second alternative may be related to the early development of the system. In northern Newfoundland, an early Pre-Middle Ordovician sequence of island arc volcanics¹⁶ was developed above a subduction zone^{17,18} dipping to the east. These volcanics thin the South-west and, in central Newfoundland, both island-arc volcanics and garnetiferous leucocratic granites are present (B. F. Kean, unpublished). Further to the Southwest, the volcanics disappear along the trace of the Cape Ray Suture. Garnetiferous leucocratic granites are, however, present in south-western Newfoundland.

The absence of the volcanics indicates either that they were obducted along the suture or else they were never developed in south-western Newfoundland. If they were removed by obduction there should be a great increase in intensity of deformation towards the suture. This does not occur (B. F. Kean, unpublished). Thus the volcanics were never developed in southwestern Newfoundland. Subduction did, however, occur in this area, as indicated by the presence of garnetiferous leucocratic granites to the South-east of the suture.

This southwards thinning of the island arc volcanics and incoming of garnetiferous leucocratic granites can best be explained by an asymmetric subduction event, that is, the subduction zone was not parallel to the continental margin. In northern Newfoundland subduction occurred dominantly under oceanic crust. In central Newfoundland subduction

occurred under both oceanic and continental crust. In southeastern Newfoundland subduction occurred dominantly under continental crust (Fig. 3).

In the North, the obduction event was related to a continent-oceanic crust island arc collision around Middle Ordovician times. Minor subduction continued through Silurian times, however, with the development of a late arc sequence¹⁹ of dominantly calc-alkaline volcanics. This sequence thins to the South-west and disappears along the trace of the Cape Ray Suture. In the South, continued subduction of oceanic crust from Pre-Middle Ordovician times to Late Silurian-Early Devonian times resulted eventually in the complete closure, that is, continent-continent collision, of the system. This caused ophiolite obduction, the overriding of the eastern over the western continent, the dismemberment of the ophiolite, and the development of an antithetic thrust belt.

Thus the closure of the Proto-Atlantic Ocean and the variable styles and ages of ophiolite obduction are seen as a complex sequence of events controlled by, and related to, the asymmetry of subduction with respect to the continental margins.

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Re-analysis of radiation-induced specific locus mutations in the mouse

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A re-analysis of published data on mouse mutation rates induced by X and gamma rays suggests that the kinetics of induction can be analysed by fitting the data to a parabolic curve. We interpret this to mean that a substantial proportion of the induced mutations results from gross chromosomal changes such as deletions, some of which are one-track and some of which are two-track. This analysis is based on the assumption that the shape of the dose curve, which in the female is concave upward, reflects the manner in which the mutations are induced rather than representing a one-track (linear) curve whose shape has been modified by differential repair.

MOST of the data dealing with the genetic effects of ionising radiation in mice have been obtained with a specific locus test. The data were obtained under many different experimental conditions¹⁻¹⁷ (see refs 18 and 19 for review), and form a major element in the evaluation of possible genetic effects of radiation in man^{19,20}.

The mutations have commonly been considered to be one-track events that increase linearly with dose, although the statistical errors associated with the data are so large that they can be fitted by non-linear curves²¹. Furthermore, the mutagenic efficiency of the radiation has varied with the experimental conditions: densely ionising radiations have a high relative biological effect, or RBE⁶, and sparsely ionising radiations can be less efficient if the dose is protracted over long periods or fractionated into sub-doses given at various intervals. These constitute criteria by which true point

mutations can be distinguished from two-break chromosomal effects, and it was postulated that many (if not most) of the specific locus mutations induced by X rays are two-track deletions²¹. Many of these specific locus mutations have indeed subsequently been shown to be deletions affecting more than one functional unit²². These deletions, however, have been considered to be one-track events⁸ even though the dose-effect curve obtained by irradiating female mice with acute doses of X rays is concave upwards^{8,14}.

In order to account for dose protraction and fractionation effects, the concavity of the curve obtained from females, and so on, various mechanisms have been postulated. These have included dose-dependent intracellular repair of a one-hit phenomenon⁸, cell selection²³, and cell synchronisation³.

Rather than treating an induction curve for mutations that is concave upwards as if it represented a one-track phenomenon that empirically did not increase linearly with dose, we believe that it is fruitful to treat the specific locus mutations as if they were a mixture of two-track²¹ and one-track²² events, mainly deletions.

We have fitted the data on mouse-specific locus mutations induced by X rays that were presented in Searle¹⁸ and the UNSCEAR report¹⁹, as well as more recently published data^{13,14,17}, to the curve represented by the quadratic equation,

$$Y = C + \alpha D + \beta D^2$$

where Y equals the expected yield of mutations, C equals the control rate or spontaneous yield, α equals the coefficient of induction of mutations that occur as a linear function of the dose (D), and β equals the coefficient of induction of mutations that increase as the square of the dose. This equation has been used for the induction of two-break chromosome aberrations where two separate events are required. The events may be caused by a single ionising track (linear term) or by the interaction of two independent tracks (dose-squared term), that is, the same situation we postulate for specific locus mutations.

We believe that, in many cases, with the use of this equation it is possible to make quantitative estimates of the yields of mutations that would be obtained under different regimes of irradiation. For instance curves for acute exposures will include both linear and

quadratic components, but those for chronic exposures will be linear because the chance of interaction of independent events will be negligible.

Data from irradiated oocytes

For oocytes (Table 1), the data that determine the dose-effect curve, that is, the control point and the data obtained after single acute doses of 50, 200, and 400 R, were used to compute the regression equation. Each of the four points was weighted by the reciprocal of the binomial variance and the coefficients determined by least squares analysis. All the oocyte data in Table 1 came from matings occurring within the first 7 weeks of irradiation, since Russell¹⁰ has observed that the mutation rate derived from conceptions occurring after 7 weeks is near zero for single acute exposures. Only one litter was recoverable from the 400 R series, two litters from the 200 R series and three litters (within the 7 week period) from the 50 R series.

For the female data, three different expected values are presented. These depend upon the value for the control rate used in the analysis. Out of 164,999 unirradiated gametes tested in the same laboratory in which the 50, 200, and 400 R data were obtained, there were seven mutants observed. Six of the seven mutants, however, were offspring of the same female and represented a cluster of mutant germ cells that came from a single mutation that had occurred early in development¹⁰. Russell¹⁰ has pointed out that this cluster complicates the computation of the spontaneous mutation rate in females, and he has calculated two different rates. In one case, he assumed that the chance for mutation occurring in early development, when there was a limited number of germ cells, is less than the chance for occurrence at a later time when there are many more germ cells. Under this assumption, one would expect clusters to be much rarer than single mutations and there would be little error in assuming that only two mutations were obtained. In the other case, the assumption made was that the only estimate of the frequency of clusters was that observed in the data. This leads to the use of all seven recovered mutations in computing the spontaneous mutation frequency.

Although an estimate of the spontaneous rate in females could be based on seven mutants, this estimate, for reasons first pointed out by Luria and Delbruck²⁴, would have a very large error

Table 1 Specific locus mutations from irradiated mouse oocytes

Item	Ref.	Experimental conditions	Gametes tested	Observed	Number of Mutants Expected		
					Cluster=0	Cluster=1	Cluster=6
1	10,18	Control	164,999	1,2, or 7	0.97	1.93	6.6
2	10,18	50 R Acute 250 KVP X ray	180,472	13 (6.7-21.4)	13.56	13.63	13.95
	5,18	200 R Acute 250 KVP X ray	37,297	21 (12.8-31.7)	19.07	18.85	17.75
	8,18	400 R Acute 250 KVP X ray	14,842	23 (14.9-34.0)	24.13	24.27	24.91
3	8,18	400 R Acute 250 KVP X ray (8 fractions of 50 R, 75-min intervals)	27,906	19 (11.2-29.0)	15.63	16.86	9.4
4	5,18	400 R Acute X rays (2 fractions of 200 R, 1-d interval)	6,086	9 (4.5-16.8)	6.19	6.15	5.55
5	4,18	400 R ¹³⁷ Cs (8 h.chronic αD contribution only)	20,827	7 (3.3-13.8)	8.49	7.57	3.02
6	11,18	600 R ⁶⁰ Co (12 d chronic αD contribution only)	10,117	1 (0.05-5.3)	6.16 (3.1)	5.45 (2.8)	2.0 (1.27)
7	10,18	258 R ¹³⁷ Cs (20 d chronic αD contribution only)	8,373	1 (0.05-5.3)	2.22 (1.13)	2.0 (1.0)	0.9 (0.62)
8	10,18	400 R ¹³⁷ Cs (31 d chronic αD contribution only)	29,325	3 (0.82-8.1)	11.91 (3.28)	10.69 (3.08)	4.27 (2.00)
9	14	200 R Acute X rays ($\alpha + \beta$, three litters, half of matings immediately after irradiation, half 1 week later)	34,813	9 (4.5-16.8)	17.78	17.66	16.59

The regression analyses were calculated when the spontaneous cluster was counted as contributing 0, 1, or 6 mutations to the control and is based on the four data points in items 1 and 2. These regression coefficients were used to predict the values in items 3-9. The numbers in parentheses are the expected values for the estimated effective dose (see text) delivered to maturing oocytes assuming that some of the irradiation was received by cells in insensitive stages. The numbers in brackets are the 95% confidence limits for the observed manner of mutations⁴⁴. Ref. 18 is a comprehensive review.

Table 2 Mutation coefficients calculated from data from spermatogonia and oocytes

	Male	Female Cluster counted as			
		0	1	6	
C	8.30×10^{-6}	8.39×10^{-7}	1.67×10^{-6}	5.74×10^{-6}	
α	6.88×10^{-8}	1.43×10^{-7}	1.26×10^{-7}	3.76×10^{-8}	
β	6.57×10^{-10}	1.09×10^{-9}	1.14×10^{-9}	1.37×10^{-9}	
α/β	104 R	131 R	111 R	275 R	

α expressed as mutations per locus R^{-1} ; β expressed as mutations per locus R^{-2} ; C is the control frequency; α/β equals the dose where the linear contribution (αD) equals the two-hit contribution (βD^2). To fit the coefficients to the data presented in Tables 2 and 3, multiply the coefficients by seven, the number of loci examined.

associated with it. Another way of treating the spontaneous rate is to make use of the fact that any cluster has to arise in pre-oocytic germ-cell stages. Since only oocytes were irradiated in the treated series, we are really concerned in our analysis only with spontaneous mutations arising in such cells. At most, one spontaneous mutation arose in oocytes sampled in the control series. As far as the treated series are concerned, no clusters were reported. Of course, single mutational events may also have arisen in oogonia, but this is true for both the irradiated and control series. For these reasons we believe that, in the present context, the best estimate of the control rate is based on only one mutant ignoring the cluster of six.

In Table 1 we present expected values based on all three estimates of the control rate.

The coefficients for the equation are given in Table 2 for all three fits. From these coefficients the expected number of mutants for other experiments was calculated. For items 3 and 4 (fractionated doses) of Table 1, the equation was used to determine the expected value for each component of the treatment and the effects were then added. For items 5–8, where the treatment was chronic (a dose rate of less than 1 R min^{-1}), only the linear component ($\beta=0$) was used, since the linear term should be independent of any dose rate effect. The agreement between predicted values and observed values when the cluster was either disregarded or counted as one mutation is excellent except for the last three items where, we believe, the period of irradiation was so long that the recovered oocytes were to a large extent irradiated in a variety of developmental stages including the most immature and less mutable ones (stages 2, and probably 3 of Pedersen²⁵).

Although many mouse strains have a 4–6-d oestrous cycle in which oocytes routinely mature and ovulation occurs, this can be readily modified by both endogenous and exogenous factors²⁶. For instance, the maturation of oocytes can be affected by pregnancy and by crowding the mice into all-female groups free from the scent of males (which suppresses oestrous) as well as by radiation (which induces superovulation²⁸ that can last for up to 16 d during which even immature oocytes can be ovulated²⁹). Thus, when radiation is administered over a prolonged period and the animals subsequently mated for 7 weeks, it is difficult to determine how much dose was accumulated in any given stage of oocyte development.

Precise knowledge of the stage of the oocyte at the time of irradiation is important because (1) the radiosensitivity varies with the stage and (2) oocytes sampled after acute exposure are different from those sampled after chronic exposure.

As regards the first, primordial oocytes (stage 2 of Pedersen) are killed far more readily by acutely delivered X rays than are mature oocytes³⁰. Paradoxically these primordial oocytes are the ones reported to have a near zero induced mutation rate with either low or high linear energy transfer (neutron) radiation delivered either acutely or chronically¹⁰. The reasons for the mutational insensitivity of these immature oocytes are obscure, although Russell has listed three possible explanations: (1) the immature oocytes are resistant to radiation damage, (2) their repair capacity

is greater, (3) the mutant cells are selectively eliminated. He favours the view that they have a greater capacity for repair. We note, however, that chronically irradiated female mice produce 60–70 offspring throughout their reproductive lifespan³¹. There are many thousands of immature oocytes in the ovary and over 4,000 are normally committed to further development, but not necessarily ovulation²⁵. Thus, about 98% of the cells are in some way eliminated, leading us to believe that selection against the mutants occurs.

As regards the sampling of different oocytes after acute and chronic exposures, we note that after acute exposures, in which the radiation was delivered in a few minutes, the majority of the matings that produced the first litters occurred within a few days of irradiation. Thus, the first litters came from oocytes that were in the most mature, and presumably the most mutable, stages. The second litters presumably came from oocytes that were in less mature stages. The third litters, which were produced only in the 50 R series, should have come from oocytes that were immature at the time of treatment^{25,27}. Unfortunately the data for the separate litters are not published, but it is likely that our mutation rate estimates after acute exposures are somewhat reduced because of the non-uniformity of the stages sampled.

In contrast, during chronic exposures lasting 12–35 d, the females may have had as many as nine oestrous cycles intervening between the inception of irradiation and the time of mating. Clearly then the oocytes providing the first, and particularly any subsequent, litters in the chronic series were in a different stage for most of the irradiation than their counterparts in the acute series.

Thus for all these aforementioned reasons, we think it is likely that the small numbers of mutants observed in items 6–8 are the consequence of some of the oocytes having been irradiated during the less mutable stages rather than resulting from repair of pre-mutational damage in a static cell population. The effective dose received by sensitive stages would then be less than the total dose delivered. We have attempted to assess the effective dose resulting from protracted irradiation on the simplifying assumption that there were ongoing oestrous cycles at 4-d intervals during the exposure. Thus if, as in item 6, the irradiation is protracted over 12 d, which is the transit time from stage 3b to ovulation²⁵, most of the cells recovered from a mating occurring immediately after the irradiation would have been mutation-insensitive immature cells at the beginning of irradiation. Approximately 4 d after the irradiation was initiated these cells would have moved into the maturing oocyte compartment to replace those which had ovulated. Their effective dose then would have been 8/12 of the nominal dose delivered. In this experiment, however, while most of the matings took place within 3 d of the irradiation, some of the females in fact may have been mated over a period of 2 weeks¹¹, and several more oestrous cycles may have occurred before fertilisation. The consequence of this would be to provide oocytes that received only 4/12 to 0/12 of the dose while they were in stages 4–7 (the fraction would depend on the number of intervening ovulations). A conservative assumption would be that the progeny examined (all first litters) came from oocytes that received between 8/12 and 4/12, or an average of 6/12, of the nominal dose. This should lead to an expected number of 2.8 mutants for this experiment, or to an even smaller number if there were more ovulations or superovulations intervening between irradiation and the mating. Similar calculations were made for items 7 and 8, and the expected numbers appear in parentheses in Table 1.

These crude corrections bring the expected numbers into reasonable agreement with the data. We do not argue that these corrections are appropriate, but our present ignorance of the effects of irradiation on the early oocyte stages does not justify a more elaborate treatment. For the present, however, we believe that it is reasonable to assume that the discrepancy in items 6–8, where the irradiation extended over many days, is caused by some of the radiation occurring during less mutable stages.

Item 9 shows recent data from another laboratory¹⁴. Seven of the nine mutants appeared in the first litter and only two in the second. We have no specific explanation why the yield here is

Table 3 Specific locus mutations from irradiated mouse spermatogonia

Item	Ref.	Experimental conditions	Gametes tested	Number of mutants	
				Observed	Expected
1	18	Control	688,921	39 (27.7–52.2)	40
2	4,9,18	300 R Acute X rays (used to compute β)	103,755	64 (48.7–80.3)	64
3	4,18	600 R Acute X rays (α and β)	119,326	111 (90.4–132.0)	239.1
a	12,13,18	670 R Acute X rays (α and β)	11,138	12 (6.7–20.3)	27.2
b	13,18	636 R Acute ^{60}Co γ rays (α and β)	12,021	11 (5.3–19.1)	26.8
c	15,18	600 R Acute ^{60}Co γ rays (α and β)	44,352	33 (22.9–45.3)	88.8
4	5,18	600 R Acute X rays (100 + 500, α and β 1-d interval)	24,811	42 (30.0–55.5)	38.6
5	13,18	626 R Acute ^{60}Co γ rays (α only, 60 daily doses 10 R)	23,982	7 (3.3–13.8)	8.6
6	13,18	600 R Acute X rays (12 $\times$ 50 R, α and β , 1-week intervals)	18,119	16 (9.6–25.4)	8.8
7a	5,18	1,000 R Acute X rays (α and β)	44,649	29 (19.1–40.9)	229.5
b	4,18	1,000 R Acute X rays, 2 $\times$ 500 R (α and β , 2-h interval)	14,879	12 (6.7–20.3)	76.5
8a	5,16,18	1,000 R (2 $\times$ 500 R, α and β , 1-d interval) Acute X rays	16,626	55 (40.9–71.1)	47.2
b	17	1,000 R (2 $\times$ 500 R, α and β , 4-d interval) Acute X rays	7,168	11 (5.3–19.1)	20.4
c	17	1,000 R (2 $\times$ 500 R, α and β , 7-d interval) Acute X rays	8,271	11 (5.3–19.1)	23.5
d	4,15,18	1,000 R (600 + 400, α and β , 15-week interval) Acute X rays	4,904	10 (5.3–17.6)	14.4
9a	4,18	1,000 R (5 $\times$ 200, α and β , 1-d intervals) Acute X rays	8,588	16 (9.6–25.4)	12.5
b	4,18	1,000 R (5 $\times$ 200, α and β , 1-week intervals) Acute X rays	10,968	15 (8.1–23.8)	16.0

Numbers in parentheses are the 95% confidence limits for the observed number of mutations⁴⁴.

lower than for the same dose (200 R) in item 2, but it has been suggested¹⁴ that the Harwell strain has possibly diverged from the Oak Ridge strain during their years of separation. (We think it most unlikely that the difference could be attributed to a dose rate effect¹⁴, since in item 2 the time for irradiation was approximately 2 min and in item 9, approximately 4 min).

Data from irradiated spermatogonia

For spermatogonia (Table 3), there was not a sufficient number of points at low doses to establish the shape of the dose curve. Therefore, to obtain the coefficients of mutation induction we adopted a different procedure than was used for irradiated females. The point at the lowest dose (300 R) was emphasised because differential cell killing and selection^{3,23} grossly distort the shape of the curve at high doses, for example, after 1,000 R fewer mutations are obtained than after 600 R. For the male, the linear regression coefficient, α , was obtained from a weighted linear regression analysis of all the extensive low dose rate data presented in Lyon *et al.*³⁵ and the control data¹⁸. The control data are given as item 1 in Table 2; the data summarised by Lyon *et al.* are not shown. This estimate of α , the control data, and the data obtained at 300 R (item 2) were used to calculate the value of β . The expected numbers were obtained from the equation as they were for the female data. All irradiation at less than 1 R min⁻¹ was assumed to be chronic and only the linear term was used to obtain expected values. The agreement is generally good for acute irradiation and for fractionated doses where individual doses are less than 500 R. The gross discrepancies at high acute doses, as noted before, are attributable to differential cell killing and selection. It is particularly noteworthy that induced rearrangements reflect a similar pattern in this dose range^{17,18}.

The agreement is reasonably good except in two circumstances: (1) when the nominal dose instead of the effective dose is used to calculate the effect in females that were irradiated over long periods that extended into the early genetically insensitive stages, and (2) when high doses of acute irradiation, which cause differential cell killing or cell selection, were given to males.

This analysis leads to the suggestion that the similar kinetics observed for the induction of specific locus mutations and gross chromosomal aberrations is not fortuitous, but a reflection of both their common mode of origin and their subsequent common

response to modifying physiological factors. For instance, the analysis indicates that independent ionisations only interact to give a dose square term within a few hours, as has previously been found for chromosome breaks in other systems^{36,37}. Such breaks have been found to interact with one another over distances of the order of 1 μm . Reported values are 1.8–2 μm ⁴⁰, and 0.2 μm ⁴¹. An analysis of the size of the site within which the two events must occur to give a specific locus mutation, can be carried out by utilising the method of Kellerer and Rossi³⁹. This relates the target size to the dose where the linear and quadratic components are equal, that is, where αD equals βD^2 . This dose is given by c/β . In Table 2 we see the value of α/β is 104 in the male and is 111, 131, or 274 in the female depending upon which value for the control was used in the calculations. A corresponding value for the diameter of the site can be obtained directly from Fig. 5 of Kellerer and Rossi³⁹. The target radius is thus found to be approximately 0.55 μm , which is very close to the value obtained for chromosome aberrations⁴². This strengthens our belief that the specific locus mutations are indeed deletions. Moreover, Valcovic and Malling (personal communication) have been unable to induce point mutations that would be recognised as electrophoretic variants at nine protein-determining loci in mouse spermatogonia, although they have recovered a number of "null" mutants which, pending further analysis, they consider to be deletions.

The present method of analysis has heuristic value and highlights some mouse experiments that could be performed to distinguish this model from that invoking the repair of pre-mutational damage (and a dose or dose-rate dependent repair system that affects the shape of the curve). For instance, in the female, chronic irradiations lasting only 4–5 d at doses similar to those employed in the acute experiments, with matings restricted to one litter, should eliminate the problem of contamination by cells that were in the insensitive stage for a considerable portion of their radiation history. A simultaneous large scale control experiment in the female would help establish a more accurate spontaneous rate. Because it becomes critical to delimit the mutation rates for the different stages of oocytes, one other area of investigation that becomes highlighted by the present analysis is that of timed conceptions after female irradiations. In the male, experiments could be performed that would encompass acute

irradiation in the range of 100–500 rad. It is over this dose range that there would be a lesser tendency for extraneous factors to obscure the shape of the dose curve. This dose range, in addition, would provide information that would be useful for interpreting the saturation and differential cell killing phenomena encountered at higher doses (600 R to 1,000 R). We believe that the 300 R dose point listed above should be included because it is the point in common with previous experiments and could be used to ensure reproducibility of results.

Estimates of the mutation rate in mice have been used to determine the genetic hazards of radiation to humans^{19,20}. We believe that the values of α represent the mutation rate per rad to be expected under conditions of chronic irradiation where the two-hit component becomes negligible. For males this is the same value as obtained by a linear fit to the chronic data, the usual basis for human risk estimation.

Because the mutation rate obtained in mouse females drops to near zero (3/259,683) after the first three post-irradiation litters, when only eggs that had been irradiated as immature oocytes are sampled, it is often assumed²⁰ that in humans, too, chronic irradiation of the female over the whole pre-reproductive lifetime mainly will reach less mutable stages. Therefore the radiation-induced rate for females has been taken as essentially zero, and the overall risk from irradiation of both sexes has been taken as half the male mouse rate²⁰. It is unknown, however, whether or not the mutational response of the human oocyte is the same as that of the mouse. There is evidence for one effect of radiation, cell killing, showing that humans and other mammals differ from the mouse^{19,30}. In view of this, we, in agreement with the view expressed by Searle and Phillips⁴³, believe that the prudent, or conservative approach to take in estimating genetic risks is to assume that the mutations do not drop to zero in human females. This will tend to make the estimate of the risk somewhat higher, which, in view of the uncertainties involved, we believe to be the more responsible way to establish radiation safety standards.

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Spontaneous mutation by mutagenic repair of spontaneous lesions in DNA

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Strains of yeast carrying mutations in many of the steps in pathways repairing radiation-induced damage to DNA have enhanced spontaneous mutation rates. Most strains isolated because they have enhanced spontaneous mutation carry mutations in DNA repair systems. This suggests that much spontaneous mutation arises by mutagenic repair of spontaneous lesions.

WORK from the laboratories of Bessman¹ and Nossal², following the work, for example, of Speyer³ and Drake and Allen⁴, has established the idea that much spontaneous mutation in *Escherichia coli* and its phages arises by mistakes made by a polymerase/exonuclease complex during DNA replication. The 3'-exonuclease seems to have an editing function, scanning newly synthesised DNA for errors made by the polymerase, and variation in the amount of editing by the exonuclease is related to the frequency of spontaneous mutation.

In an analysis of mutants selected for mutator activity in

the yeast *Saccharomyces cerevisiae*, we have become aware of the inadequacy of the editing hypothesis, for these mutations occur at too many loci. Eight loci are definitely established, there is partial evidence for four more, and the system is clearly not saturated.

Induction of spontaneous mutations by natural agencies has been discussed ever since Muller⁵ and Stadler⁶ showed that X rays cause mutation. Intracellular production of mutagens⁷ to explain mutators⁸ and spontaneous occurrence of tautomeric changes in bases of DNA⁹ have also been suggested.

After the discovery that yeast had a higher spontaneous mutation rate in meiosis than in mitosis¹⁰, the possibility that errors were implicated in the other DNA metabolic processes, such as recombination¹¹ and replication^{12,13}, became widely accepted.

Ultraviolet and X-ray mutagenesis in *E. coli* require an intact repair system^{14–17}. The same applies to yeast for ultraviolet light^{18,19}, implicating the *RAD6* pathway, and for several chemical mutagens^{20,21}, again implicating the *RAD6* pathway, as well as *rad15*, which does not affect ultraviolet mutagenesis²².

Table 1 Mutator activity of *rad6-1* and *rad51-1*

(a) Tetrads segregating for <i>rad6-1</i>				
Tetrad no.	Reversion of <i>lys1-1</i> in UV-sensitive segregants		Reversion of <i>lys1-1</i> in UV-resistant segregants	
1	92	70	46	32
2	72	110	47	46
3	116	113	39	32
4	88	100	59	48
5	133	128	47	29
6	90		48	31
7	140	116	32	47
8	124	154	24	28
9	93		28	47
10	112	131	27	35
21	76	113	23	43
22	95	124	46	30
23	123	115	24	29
24	108	131	30	25
25	107	113	19	34
Mean of 28 <i>rad6-1</i> strains with standard deviation 110.24 ± 20.45			Mean of 30 <i>RAD6</i> strains with standard deviation 35.83 ± 10.14	
(b) Random meiotic products from tetrads segregating for <i>rad51-1</i>				
	Reversion of <i>lys1-1</i> in UV-sensitive segregants		Reversion of <i>lys1-1</i> in UV-resistant segregants	
	148		88	
	151		29	
	122		46	
	154		20	
	156		16	
			41	
			33	
			36	
			19	
			31	
			35	
Mean of 5 <i>rad51-1</i> strains with s.d. 146.20 ± 13.86			Mean of 11 <i>RAD51</i> strains with s.d. 35.82 ± 19.64	

The values given are the numbers of lysine-independent colonies arising during growth on plates with limiting lysine content³⁸. Each value is the average of two or more determinations, corrected for previously existing revertants. The method has been standardised with the fluctuation test method of determining reversion rates³⁹.

Study of the accumulated data on radiation sensitive and mutator mutants in yeast in the context that induced mutation is a repair phenomenon, reveals that spontaneous lesions susceptible to mutagenic repair are occurring in yeast.

Working model of repair processes in yeast

Radiations and many chemicals interact with DNA to produce a molecule which is locally not DNA. This chemically changed part of the DNA is known as a primary lesion. The primary lesion is a substrate for several systems of repair enzymes which recognise the distortion of the molecule and restore chemically sound DNA. In the case of ultraviolet-induced pyrimidine dimers it has been shown that one unrepaired lesion is a lethal event^{23,24}. We assume that a chemical alteration of DNA interferes with replication and/or function of the genetic material, and that most types of lesion are lethal.

The problem for repair systems is to know how to restore the informational content of the DNA. Photoreactivation of dimers is a chemical reversal of the lesion, so that information is never lost. Excision repair removes a length of the nucleotide chain containing the lesion and uses the complementary chain as a template for resynthesis^{35,26}. In some situations this solution is not available, perhaps because that enzyme system is already used to capacity, because of the configuration of close lesions, or because of the type of lesion involved. There is evidence for bacteria that replication past the site of a dimer makes it no longer amenable to excision repair¹⁶.

In the cases not amenable to excision repair, the information may be taken from a sister chromatid or homologue. The effects of this may be seen genetically as induced crossing over or gene conversion. A further type of repair may use no record of the damaged information, or use one which cannot be copied accurately, so that the chemically sound DNA which is restored differs in its informational content from that which was originally damaged. The expression "error-prone repair"²⁶ is widely used for this kind of repair, but we prefer to call it "mutagenic repair" to avoid the implication that wrong information was inserted by mistake rather than as a necessity.

It is repair that causes the broad shoulder on the ultraviolet survival curve of wild-type yeast²⁷. At low doses there is very little cell death because all lesions are repaired by one or another repair pathway. At higher doses the repair capacities become saturated or inactivated, or are no longer capable of being induced^{28,30}, so that the killing curve becomes more nearly exponential as the kill becomes proportional to the induction of primary lesions.

Channelling of lesions

When a repair pathway is blocked, the lesions which would have been repaired by that pathway may be handled by the other repair capacities^{31,32}. If the pathway which is blocked is not a mutagenic repair pathway, it is to be expected that the strain will show a higher frequency of induced mutation per exposed cell at a given dose, because a higher proportion of the lesions is channelled into mutagenic repair.

It may be expected that all blocks affecting a first step in a pathway can channel lesions into other pathways, for the lesions remain unrepaired. If, however, the block is at an intermediate step in a repair pathway, the partially repaired lesion may not provide a suitable substrate for other repair capacities. This irreparable secondary lesion will then be lethal. In this situation, the kill will vary as the number of primary lesions accepted by the deficient pathway. Radiation-sensitive strains of this type will not be able to channel lesions into mutagenic repair. (They may, however, show an enhanced induced mutation frequency per survivor because the survivors were those cells which did not use the blocked pathway.)

It has been shown for yeast^{33,34}, and several other organisms³⁵, that some radiation-sensitive strains are mutators, while others are not. If radiation-sensitive strains derive their mutator activity from channelling spontaneous lesions into mutagenic repair pathways, it is to be expected that first steps in pathways would all be able to channel lesions, and therefore be mutators.

There are three first steps of repair pathways for ultraviolet-induced lesions in yeast³⁶, *rad3*, *rad51* and *rad6*. Brychcy found³⁷ that *rad3* was a mutator, while later steps in the excision repair pathway³⁶ (*rad1*, *rad2* and *rad4*) were not. Table 1 shows that *rad51* and *rad6* are also mutators. Thus blocks in all three first steps cause enhanced

Table 2 Sensitivity of mutator mutants at eight loci to the lethal effect of three mutagenic agents

	X rays	UV	MMS*	No. of alleles tested
<i>mut1</i>	R	R	R	6
<i>mut2</i>	R	R	S	11
<i>mut3</i>	R	WS	S	2
<i>mut4</i>	R	WS	S	1
<i>mut5</i>	S	WS	S	1
<i>MUT6</i>	R	R	R	1
<i>mut9</i>	WS	WS	S	1
<i>mut10</i>	S	R	S	1

R, wild-type level of resistance; S, sensitive; WS, weakly sensitive; MMS, methyl methanesulphonate.

*These results were obtained by Anwar Nasim and Theresa Brychcy.

spontaneous mutation, while blocks in later steps may or may not show mutator activity.

These observations lead to the conclusions that all three pathways are involved in the repair of spontaneous lesions, and that when any of these pathways is unable to "accept" a lesion there is an increased probability of the lesion being repaired by a mutagenic repair pathway.

It is especially interesting that *rad6* is a mutator because this is the first step of the pathway responsible for most ultraviolet and X-ray-induced mutations²², and for most or all mutations induced by a variety of chemical mutagens²⁰. We interpret this to mean that another mutagenic pathway repairs spontaneous lesions and leads to mutation with a higher efficiency than the *RAD6* pathway. The alternative interpretation, that the *rad6* mutant is leaky, and mutated in such a way that it is more likely to make mistakes, is discounted because the *rad6-1* allele used is amber suppressible and therefore unlikely to have any function in the mutant protein. Lawrence *et al.*¹⁹ showed that *RAD6* is predominantly responsible for transitions, which implies that another pathway causes other types of mutation.

Sensitivity of mutator strains and recessiveness of mutations

Since repair pathways show little specificity in the types of lesion on which they act, it is to be expected that strains with blocks in pathways involved in the repair of spontaneous lesions will show sensitivity to lesions induced by mutagenic agents. If mutator mutations act by channelling spontaneous lesions into mutagenic repair pathways, it should be possible to confirm that the genes are involved in repair of damage to DNA by identifying the sensitivity of the mutants to mutagenic agents.

The results obtained so far are summarised in Table 2. Only *mut1* and *MUT6* fail to show sensitivity to one or more of the agents tested.

Loss of a repair capacity is expected to be recessive, and the radiation sensitivity of *rad* mutations has been found to be recessive⁴⁰. In contrast, it is to be expected that genetic control of the frequency of errors made during DNA synthesis will be incompletely dominant. This is clearly the case if the lesion is in the polymerase itself. If the mutator mutation affected an exonucleolytic edition function¹, the mutator activity would still be incompletely dominant if the 3'-exonuclease were a part of the polymerase. Recessiveness of such an altered exonuclease function could be found only if the wild-type exonuclease were free to scan all replication sites, and the mutant exonuclease were absent.

For these reasons we take the recessiveness of a mutator mutation to be strong support for the idea that it acts by modifying the repair capabilities of the cell, and not by changing other DNA metabolic processes.

Table 3 shows determination of dominance for the mutator mutations. Most mutators are less effective in a homozygous diploid than in a haploid. We attribute this to a mode of repair present in a/α diploids and absent from haploids. It can be said that all alleles tested at seven of the eight loci are recessive, and that *MUT6* shows dominance in some backgrounds.

Implications

The argument presented here can be summarised as follows. Radiation-sensitive mutants show induced mutation frequencies which differ from those found in the wild type because the mutations cause changes in the proportions of primary lesions which are channelled into various repair pathways. Some of the repair pathways are mutagenic and others are not.

That these same radiation-sensitive mutations affect the frequency of spontaneous mutation is taken as evidence that spontaneous lesions occur, and are repaired by systems having many steps in common with the systems repairing

induced lesions. It can also be concluded that mutagenic repair is available to the spontaneous lesions.

When strains of yeast are isolated which have increased spontaneous mutation rates, it is found that they represent mutations at many more loci than would be expected to be involved in DNA synthesis, that most of them are recessive and that most of them show sensitivity to mutagens. These points taken together lead to the conclusion that the mutators enhance spontaneous mutation rates by channelling spontaneous lesions into mutagenic repair pathways. The only candidate among these strains for a mutator acting on other DNA metabolic processes is *MUT6*, which is dominant and not known to be sensitive to any mutagenic agent. Another possible case was reported by Esposito *et al.*⁴¹, who found that *spo7*, which seems to be involved in premeiotic DNA synthesis, was an antimutator.

Table 3 Mutator activity in diploids homozygous and heterozygous for alleles at eight loci

Locus and allele	<i>mut</i> —	<i>mut</i> +	+
	<i>mut</i>	—	—
<i>mut1-1</i>	657	43	31
-2	761	131*	61
-4	455	34	27
-5	253	34	30
-6	417	50	30
<i>mut2-1</i>	158	55*	61
-2	274	41	61
-5	389	58	61
-6	188	28	27
-7	186	31	30
-8	139	26	30
-9	337	46	20
-10	185	23	20
-11	346	24	18
<i>mut3-1</i>	168	53	31
-2	125	55	20
<i>mut4-1</i>	96†	34	31
<i>mut5-1</i>	265	36	20
<i>MUT6-1</i>	182	51‡	18
<i>mut9-1</i>	280	32	20
<i>mut10-1</i>	84	18	19

Values given are as for Table 1. They are the average of those obtained from at least four independently isolated diploids, except in the cases noted below.

* Values determined from two independently isolated diploids.

† Value is the average of 38 independently isolated diploids. The values obtained ranged from 30 to 270, so that the mutator activity could not be detected in some homozygous *mut4-1* diploids.

‡ This value is the average of 24 independently isolated diploids. The values obtained ranged from 14 to 229, so that *MUT6-1* was seen to be dominant to the wild-type allele in some heterozygotes, and recessive in others.

An explanation of mutator activity in terms of channelling of spontaneous lesions leads to the conclusion that the frequency of spontaneous lesions is higher than the frequency of spontaneous mutations in wild-type strains, and is therefore high enough to account for all spontaneous mutation. We also conclude that mutagenic repair is not induced by the presence of mutagenic agents, but is constitutive, at least in some cells. Lemontt⁴² has published evidence that such repair is constitutive in *Ustilago*.

Whether any part of the spontaneous mutation frequency of wild-type cells can be attributed to mistakes in replication can be determined by isolating antimutator strains which have lost all mutagenic repair capacities. A further problem raised by these conclusions is the origin of the spontaneous lesions, which may be either endogenous or naturally induced. One possibility is that replication errors provide a substrate for repair, and are therefore a source of spontaneous lesions.

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Somatic mutation as the basis for malignant transformation of BHK cells by chemical carcinogens

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The chemical induction of malignant transformation in BHK cells seems to result from a somatic mutation. Stable transformants, whose frequency is significantly increased by mutagenic carcinogens, can revert to normal and often display temperature-restricted phenotypes indicative of an altered gene product.

ALTHOUGH the many alterations in cell growth, morphology and biochemistry which accompany the chemical induction of malignant transformation in cultured mammalian cells have been extensively described, the primary event by which the inducing carcinogen causes these changes is unknown^{1,2}.

Two general mechanisms have been proposed to explain how this initial cellular transformation occurs during chemical carcinogenesis. On the one hand transformation may be the result of an epigenetic change in the expression of a gene or activity of a gene product which is not due to an alteration in the primary structure of DNA. Such changes are presumed to be responsible for much of development, including the activation-inactivation of the mammalian X chromosome³ and the extinction and activation of differentiated functions which have been observed in cultured cells⁴. The ability of markers from embryonic tumour cells to reappear in normal differentiated adult tissues⁵ lends support to this idea that transformation results from a change in gene function rather than in gene structure.

Alternatively, transformation may be the result of a somatic mutation, defined here in Siminovitch's global sense⁶ as a heritable change in nucleotide sequence resulting from an alteration, deletion or rearrangement in the primary structure of cellular DNA. Most of the chemicals known to cause cancers in animals are mutagenic, their active forms interact with DNA *in vivo* and *in vitro*^{7,8} and cause mutation in bacteria (refs 9 and 10 and D. Anderson

et al., quoted in ref. 8) and occasionally in mammalian cells^{11,12}. It is not clear, however, whether the observed interactions of carcinogens with DNA reflect the mutagenic nature of the events underlying their carcinogenicity or merely their almost universal electrophilic nature¹³. To show that mutagenicity is the mechanism by which chemicals induce malignancy, it is necessary to go beyond the demonstration that carcinogens are capable of being mutagens and to show that transformation itself is due to a carcinogen-induced mutation.

In this paper we attempt to do this by investigating malignant transformation *in vitro*, using the established quasi-diploid hamster line BHK21/cl 13 and the chemical carcinogens nitrosomethylurea and 4-nitroquinoline-1-oxide. Cloned cell cultures are exposed briefly to a carcinogen, grown for 3 d in liquid culture and then assayed by plating in soft agar. Cells able to form colonies in the soft agar are considered to be malignant transformed since this characteristic of anchorage independence *in vitro* shows a complete correlation with *in vivo* malignant growth^{14,15}. Whether or not these newly induced transformed cells are the result of mutations cannot be determined directly because the usual techniques of genetic recombination, DNA physical chemistry and sequence analysis of an altered gene product cannot yet be usefully applied to cultured mammalian cells. A number of other testable criteria, however, have come to be accepted^{6,16,17} by which a newly occurring phenotype in a somatic cell can be defined as a mutation. These criteria are (1) the phenotype should arise with a low frequency spontaneously; (2) this frequency should be increased by mutagenesis; (3) the phenotype should be associated with an altered gene product, usually a protein; (4) the phenotype should be stable; (5) reversion should be low but demonstrable; (6) the phenotype should be localised on a chromosome. The data presented below demonstrate that the induction of the transformed phenotype by carcinogens in cultured BHK cells fulfils the five of these six

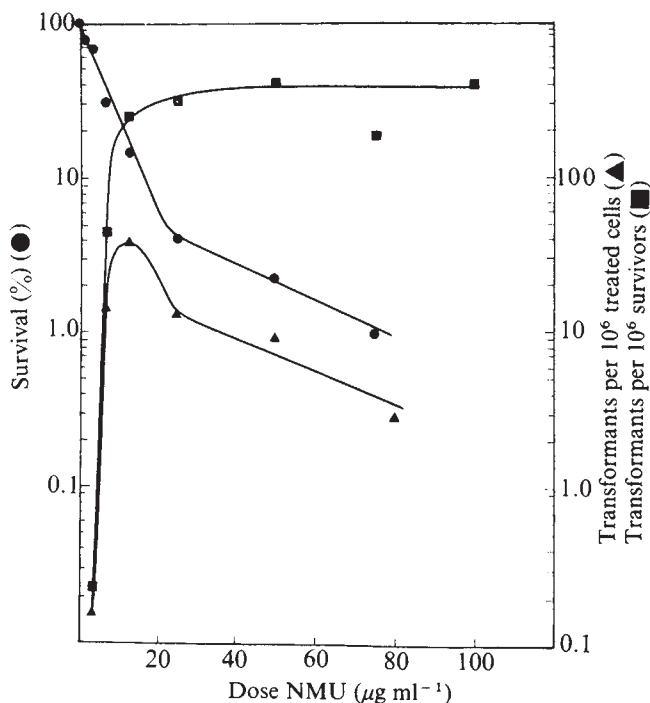


Fig. 1 Inactivation and transformation by nitrosomethylurea. BHK Supernormal cl 10 cells were washed and treated for 1 h at 37 °C with various doses of freshly prepared NMU (synthesised for these experiments) while suspended at 2×10^6 cells per ml in TD buffer¹⁵ containing 10% of the 5 mM citrate buffer (pH = 5.5) which served as carcinogen solvent. Following treatment cells were washed and assayed for survival in high serum growth media (Dulbecco's MEM + 10% tryptone phosphate broth + 20% calf serum) by measuring colony forming ability compared to that of cells treated with solvent only. There was no decrease in survival after treatment with solvent alone. Transformation was assayed following 3 d sub-confluent growth in liquid culture, during which control cells underwent six doublings, by plating in growth medium partially solidified with 0.34% agar at a density of 1×10^6 cells per 60-mm dish following the technique of Macpherson and Montagnier¹⁶. Transformants were counted as colonies growing to a diameter ≥ 0.2 mm after 2–3 weeks incubation at 38.5 °C. Concurrent assay of liquid colony formation allows one to measure the frequency of transformants per survivor at the end of the expression period. The number of transformants per treated cell was calculated by multiplying transformants per survivor times the survivors per treated cell measured immediately following carcinogen treatment.

requirements tested so far and thus seems operationally to be a mutagenic event.

Low spontaneous frequency of transformation

Normal laboratory stocks of the established line of baby hamster kidney cells, BHK21/cl 13 (ref. 18), frequently show a high background of colonies, $\leq 5 \times 10^{-4}$, when plated in soft agar. This is apparently due in part to the accumulation of spontaneously transformed variants and their enrichment when the stocks reach confluence at each passage¹⁹, and in part to a propensity for spurious colony formation at high cell densities. To avoid both these complications this line was cloned in liquid and a subline selected, BHK supernormal clone 10 (BHK SN cl 10) which clones in liquid with a normal efficiency of 50–60% but fails to form any colonies in soft agar at densities of up to 2×10^6 cells per 60 mm dish. This subline has not been tested yet for *in vivo* tumorigenicity. The frequency of spontaneous transformation (below) suggests that it would be tumorigenic at sufficiently high doses, as is its parent line¹⁸. Aliquots of this subclone were used for most experiments reported here. To minimise selection for spontaneously transformed variants, cells were not allowed to become confluent during growth.

The frequency of spontaneously arising transformants was determined at the seventh passage after recloning by assaying 10^8 untreated cells in soft agar; techniques are described in the legend to Fig. 1. Colonies arising in agar were picked, grown in liquid culture and retested in agar before being counted as transformed. No unstable transformants were observed. The frequency of spontaneously arising stable transformants was 1.3×10^{-7} per tested cell and 2.2×10^{-7} per cell able to clone in liquid. When this cloned cell line was tested earlier at fifth and sixth passage levels as controls in carcinogen experiments, no spontaneous transformants were observed (less than one transformant per 10^7 cells tested per experiment).

These frequencies of transformation underestimate the true number of transformants arising in these experiments because transformed cells do not clone with 100% efficiency in agar. The average relative plating efficiency in agar (% of cells cloning in liquid which are able to clone in agar) of the spontaneously transformed clones is 25%. When corrected for this, the spontaneous transformation frequency is 8.8×10^{-7} per viable cell. Both the observed and corrected frequencies of transformants are well within the range of frequencies observed for spontaneous mutations at well defined loci in other mammalian cells ($\sim 10^{-5}$ to $< 0.5 \times 10^{-7}$)^{17,20,21} and thus compatible with their origin as random mutations.

Carcinogens increase transformation frequency

When aliquots of the BHK SN cl 10 line are treated with either of two mutagenic carcinogens, the frequency of transformed cells is dramatically increased. Figure 1 illustrates the inactivation and transformation induced by nitrosomethylurea (NMU), a powerful carcinogen²² which spontaneously produces DNA alkylating intermediates²³ and is a base-changing mutagen in bacteria^{9,10}.

The survival curve for NMU is consistently biphasic,

Fig. 2 Inactivation and transformation by 4-nitroquinoline-1-oxide. BHK Supernormal cl 10 cells were washed, treated and assayed as described in Fig. 1 except that the carcinogen was NQO (ICN Pharmaceuticals, Plainview, New York); its solvent, DMSO, was present during treatment at a concentration of 1%; and cell concentration during exposure to carcinogen was 1×10^6 ml⁻¹.

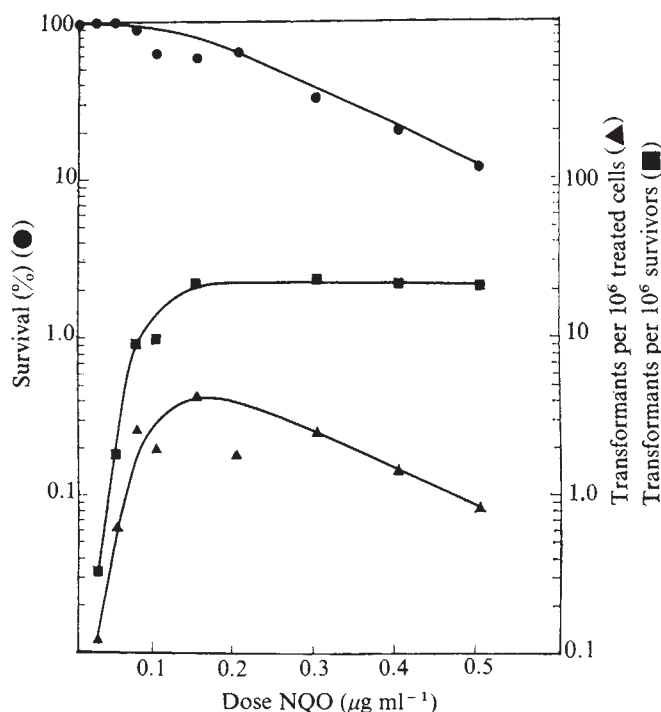


Table 1 Expression of the normal phenotype by clones of BHK SN c1 10 transformed by different doses of nitrosomethylurea

Dose NMU ($\mu\text{g ml}^{-1}$)	Number of clones in which normal phenotype is		
	Cold-sensitive	Heat-sensitive	Unexpressed
6.25	1	3	2
12.5	0	4	3
25	3	0	3
50	0	2	9
75	1	4	3
Total	5	13	20
% of total tested clones	13%	34%	53%

Transformants were isolated as individual clones arising in agar following the carcinogen treatment outlined in Fig. 1. Several colonies from each dose were grown in liquid and recloned in either liquid or soft agar at 38.5 °C. Following this second cloning, cells were split, grown in log phase at either 32 °C or at 38.5 °C for a minimum of 7 d to eliminate phenotypic lag¹⁵ and assayed at the same temperature for ability to clone in high serum liquid growth media and in growth media solidified with 0.3% agar⁵⁶. 800 total cells were tested in liquid, 10⁴ cells in agar. A clone was considered to be heat sensitive for the expression of its normal phenotype if its absolute plating efficiency in soft agar (agar colonies per cell plated) at 38.5 °C was more than 10 times greater than that at 32 °C and if its relative soft agar plating efficiency (agar colonies per cell able to clone in liquid) was more than seven times greater at 38.5 °C than at 32 °C. A clone was considered to be cold sensitive for expression of the normal phenotype if the converse of these criteria was met. The clone was considered transformed and the normal phenotype unexpressed at either temperature if the absolute agar plating efficiencies were more than tenfold greater than the normal controls and differed by less than tenfold at the two temperatures and the relative agar plating efficiencies differed by less than sevenfold.

perhaps reflecting killing by the cyanate ion breakdown product of NMU²⁴ as well as by macromolecular alkylation. NMU transforms efficiently; the maximum transformation frequency observed is 3.8×10^{-5} transformants per treated cell, 4×10^{-4} transformants per survivor, an almost 300-fold increase over the spontaneous frequency. Correcting for the 15% average relative agar plating efficiency of isolated and purified NMU-induced transformants, the frequency per survivor becomes 2.2×10^{-3} .

Killing and transformation induced by 4-nitroquinoline-1-oxide (NQO) is illustrated in Fig. 2. NQO is a free radical carcinogen²⁵ which is activated by cellular enzymes to intermediates which form NQO-purine adducts in DNA^{26,27}. It is an effective mutagen in bacteria where it causes both base changes and reading frame shifts^{9,10,28} and in yeast where it shows a specificity for GC pairs²⁹. NQO lesions undergo extensive repair in bacteria^{26,27} and mammalian cells³⁰, which is reflected in the shoulder of the survival curve.

NQO transforms effectively, even at doses where survival is unaffected. The maximum transformation frequency observed is 4×10^{-6} per treated cell, 2.2×10^{-5} per survivor, a 30-fold increase over the spontaneous frequency. If corrected for the 25% average relative agar plating efficiency of isolated NQO-transformed clones, the frequency per survivor becomes 8.8×10^{-5} .

This frequency is lower by two orders of magnitude than that observed by Kakunaga when he used similar doses of NQO to transform a BALB/3T3 mouse cell line^{31,32}. His higher frequencies may be due in part to the use of transformed foci as an assay for transformation. Although he finds that five of the focus-derived NQO-transformed lines clone in soft agar and are tumorigenic³³, we have found only one in 10 morphologically transformed colonies to be truly transformed by these criteria¹⁹, and others also find that morphological conversion can occur without tumorigenicity¹⁴.

Transformation observed in response to these two carcinogens does not appear to result from selection of pre-existing transformants which are more resistant to the inhibiting or lethal effects of the carcinogens, since (1) cells are treated with the carcinogens only briefly while sus-

pended in buffer under non-growing conditions so no selective overgrowth is possible while the carcinogen is present; (2) transformation is observed at a non-toxic concentration of NQO; and (3) the frequency of transformants per treated cell falls in parallel with survival indicating that transformed variants are as sensitive to inactivation by the carcinogens as the normal cells. In the case of NMU, this parallel is obscured at doses less than $15 \mu\text{g ml}^{-1}$ by the rapidly increasing number of induced transformants.

An expression time of about six cell generations is allowed between carcinogen treatment and assaying for induced transformants, to give time for fixation of any possible pairing errors and for phenotypic expression of transformation. Temperature shift experiments with BHK cell lines temperature sensitive for normal growth have shown that this expression requires 3.5 generations¹⁵. It is doubtful that the cell population becomes enriched for transformants during this time, as transformed lines grow with the same doubling time as normal BHK cells¹⁹, and confluent conditions where they might be at an advantage are scrupulously avoided.

At lower doses where transformation frequency per survivor rises with increasing carcinogen dose, the dose-response relationship is linear. A levelling off of the frequency of transformants occurs at higher doses, as has been observed previously for NMU³⁴ and for other carcinogens³⁵. The maximum frequencies of transformation observed are within the range of frequencies with which mutations are induced by mutagens at other, better characterised loci in mammalian cells^{20,21,34}. For example, more than 9.7×10^{-4} 8-azaguanine-resistant mutants per survivor are induced by NMU in Chinese hamster line³².

Induction of temperature-dependent transformants

This laboratory has previously reported the isolation of several chemically transformed derivatives of BHK which were selected for growth in soft agar and shown to be malignant *in vivo* yet *in vitro* behaved as normal cells at 32 °C and as transformed only at 38.5 °C (ref. 15). They were considered to have a heat-sensitive lesion in some gene product necessary for normal growth rather than a cold sensitive lesion in a product causing transformation, because they occur at low frequency and in the first case the change from the normal phenotype can be accomplished in one step, whereas in the second case two steps are required—the turning on of whatever is required for expression of transformation plus a second event to make transformation cold sensitive. This convention will be followed here.

In the current series of experiments transformed colonies resulting from the carcinogen treatments depicted in Figs 1 and 2 were selected, purified by recloning, and split to

Table 2 Expression of normal phenotype by clones of BHK SN c1 10 transformed by different doses of 4-nitroquinoline-1-oxide

Dose NQO ($\mu\text{g ml}^{-1}$)	Number of clones in which normal phenotype is		
	Cold-sensitive	Heat-sensitive	Unexpressed
0.05	0	3	1
0.075	0	3	4
0.10	0	1	6
0.15	0	6	2
0.20	0	4	4
0.30	0	1	6
0.40	0	4	1
0.50	0	2	5
Total	0	24	29
% Total tested clones	0%	45%	55%

Transformants were isolated following carcinogen treatment as outlined in Fig. 2 and grown and tested for the expression of their normal phenotype as described in Table 1 except that all reclonings were done in soft agar (0.34%).

high and lower temperatures where they were grown and tested for the expression of the normal phenotype assessed as a significant depression in absolute and relative plating efficiency in soft agar (Tables 1 and 2). At almost every dose some clones remain fully transformed at both temperatures. Fifty-three per cent of the NMU-transformed clones and 55% of the NQO-transformed clones display this unconditional phenotype. The remainder of clones tested express the normal phenotype at one of the temperatures and the transformed phenotype at the other. The majority of these temperature restricted clones are transformed at the high temperature, normal at the low, as is expected since transformants were initially selected as clones growing in agar at 38.5 °C. However, at three different doses of NMU clones arise which are normal at 38.5 °C and transformed at 32 °C. No such clones cold sensitive for the normal phenotype were seen following NQO treatment. This is probably because the second cloning was performed in agar at 38.5 °C, rather than in liquid or agar as with NMU; this imposes a second selection against such variants which do not plate efficiently in agar at high temperature.

Fourteen spontaneously arising transformants were similarly tested and none were found to be temperature dependent for transformation. However, as they all come from aliquots of the same culture, they may be sibs and represent only one transformation event. Another spontaneous BHK transformant isolated earlier in this laboratory is normal at 32 °C and transformed at 38.5 °C (ref. 15).

Due to the period of growth between the removal of the carcinogen and the assay for transformation, many of the transformed clones isolated within a single dose in these experiments may also be derived from a single transformation event. However, the minimum number of clones of independent origin can be tabulated as shown in Table 3 using data from several experiments and three carcinogens. Even with this correction, 57% of the clones are temperature restricted.

The high frequency of temperature-limited phenotypes is surprising but not unique. In *Escherichia coli* 75 out of 150 independent thymine-less mutants isolated at 37 °C were temperature sensitive, requiring thymine for growth at 37 °C but not at 28 °C³⁵. In *Saccharomyces* 12 out of 26 spontaneous *ade-3* mutants were temperature sensitive³⁶. In *Drosophila*, one half of a group of newly isolated EMS-induced notch mutants were temperature sensitive and one-quarter of existing mutants at this locus are temperature sensitive³⁷. In mammalian Chinese hamster cells 25% of cells selected for spontaneous 6-thioguanine resistance at high temperature could grow in HAT medium at low temperature, suggesting a temperature-sensitive HPRT enzyme defect which was verified *in vitro* for three clones³⁸. Certain loci in a variety of organisms seem to have particular sensitivity for temperature-sensitive lesions; the locus or loci involved in the transformed phenotype in BHK may be one of these types.

An unusually high frequency of temperature-limited phenotypes is also often found when screening for revertants of bacterial³⁹ and mammalian^{40,41} cell mutants. If the induction of transformation is the result of an alteration in a gene product whose function is vital to the cell, then perhaps the number of sites where transforming lesions can occur is limited, as is the number of sites where a change can restore function to produce a revertant. The presence of a temperature-sensitive site among these few permissible lesions could explain the high numbers of temperature-restricted phenotypes among transformants and revertants.

Temperature- and cold-sensitive transformed clones may not be unique to the BHK 21 cell line. Clones similar in some respects to those reported here have been isolated directly from BALB/c-3T3⁴² and as mutagen-induced revertants of transformed lines of rat liver⁴³ and Chinese hamster lung⁴⁴.

In most cases carefully analysed so far, temperature-restricted phenotypes have been found to be the result of a temperature-sensitive or cold-sensitive gene product, typically a protein derived from a missense mutation⁴⁵. Thus these carcinogen-induced temperature-limited transformants are most plausibly explained as resulting from carcinogen-induced missense mutations in a gene specifying some product necessary for normal cell growth. It is particularly difficult to explain by epigenetic mechanisms the induction in the same cells, in the same tube at the same time by the same carcinogen, of three distinct transformed phenotypes as is the case for two doses of NMU (6.25 and 75 µg ml⁻¹, Table 1), whereas these situations are easily explained by mutations at three different sites in a gene or genes regulating normal growth.

Stability of transformants

The transformants described above are stable both *in vitro* and *in vivo*. Temperature-restricted clones isolated several years ago¹⁵ have been grown extensively, re-cloned in liquid and in agar and always found to retain their original characteristic temperature sensitivity for expression of the normal phenotype. One of these clones, Me₂N4 (induced by dimethylnitrosamine), plated in soft agar with an efficiency of less than 0.01% at 32 °C and 6.1% at 38.5 °C when first isolated¹⁵. In a recent re-test, a minimum of 60 generations and four years later, it plated in soft agar at 0.02% at 32 °C and 27% at 38.5 °C. A clone isolated during the current experiments, NMU no. 19, plated in soft agar with an efficiency of less than 0.03% at 32 °C and 15% at 38.5 °C at the second passage after isolation. Eight passages (~56 cell generations) later, it plated in soft agar at less than 0.03% at 32 °C and 18% at 38.5 °C. *In vivo* stability was demonstrated by Dr Arthur Soller in this laboratory with cells recovered from a tumour which had been induced in Syrian hamsters by subcutaneous injection of Me₂N4. When the tumour cells were returned to culture, they were found to retain their original phenotype—transformed at 38.5 °C, but normal and unable to clone in soft agar at 32 °C.

Reversion to normal phenotype

The frequency with which the cell line Me₂N4 (ref. 15) (which is temperature sensitive for the expression of the normal phenotype) reverts to normal following ethylmethane sulphonate (EMS) mutagenesis was determined by growing EMS-treated cells at 38.5 °C in methylcellulose containing

Table 3 Clones of independent origin

Carcinogen	Expression of normal phenotype			Total clones
	Heat-sensitive	Cold-sensitive	Un-expressed	
NMU no. clones	8	3	8	19
%	42	16	42	
DMN no. clones	2	0	0	2
%	100	—	—	
NQO no. clones	8	0	8	16
%	50	—	50	
none no. clones	1	0	1	2
%	50	—	50	
Total clones	19	3	17	39
%	49	8	43	100

Clones are considered to have originated independently if: (1) they occurred in response to different doses of carcinogen in an experiment where the similarly treated and tested control culture showed no detectable transformants or (2) they occurred at the same dose, but have significantly different phenotypes, that is, cold-sensitive, heat-sensitive or unrestricted. DMN — dimethylnitrosamine. The isolation of DMN clones was reported previously¹⁵. Of the spontaneously arising transformants, one was isolated previously¹⁵, the other during the course of this work in a large experiment on BHK SN cl 10 performed as outlined in legend to Fig. 1 but omitting the carcinogen treatment. They were re-cloned and analysed as described in the legend to Table 1.

Table 4 Selections by lethal growth in methylcellulose-FUdR

	BHK		Me ₂ N4	
	No FUdR	+ FUdR	No FUdR	+ FUdR
Plated in methylcellulose				
Total cells	1.1×10^6	1.1×10^6	1.3×10^6	1.5×10^6
Total colony formers	1.7×10^5	1.7×10^5	5.7×10^5	1.1×10^6
Recovered from methylcellulose				
Total cells	7.5×10^5	6.2×10^5	8.7×10^5	5×10^5
Total colony formers	1.0×10^5	6.4×10^4	2.6×10^5	9.8×10^3
Corrected colony formers	1.3×10^5	1.1×10^5	3.7×10^5	3×10^4
% Colony formers surviving treatment	79%	68%	65%	2.7%
% Colony formers surviving second cycle	ND	ND	ND	15%
% Colony formers surviving third cycle	ND	ND	ND	12%

Me₂N4 is a chemically transformed cell line which is temperature-sensitive for the expression of the normal phenotype; BHK is its parent line, BHK21/cl 13. Selections in methylcellulose were carried out by plating 10 ml of well-dispersed cells suspended in growth media containing 1.2% methylcellulose (Fischer, 4,000 centipoise) atop a 15 ml base layer of growth medium solidified with 0.5% agar. One-hundred millimetre bacteriological plastic dishes were used to prevent cells from growing on the sides of the dishes. Plates were incubated for 3 d at 38.5 °C in a humidified atmosphere containing 10% CO₂. Where indicated, 5-fluorodeoxyuridine (FUdR, final concentration 125 µg ml⁻¹) and uridine (final concentration 500 µg ml⁻¹) were added at the end of day 1. Treatment was terminated at the end of d 3 by adding 10 ml growth media containing thymidine (Final concn 28.6 µg ml⁻¹). After 30 min further incubation, the slurry was removed, the agar base layers scraped and washed with an ice-cold mixture of 1:1 growth media and versene (0.02% disodium dihydroxy versenate in phosphate buffer). The cells were collected from the diluted slurry, washed by centrifugation, counted and assayed for colony formation in growth media containing 20% calf serum. Corrections were made for any loss in cell numbers during recovery from methylcellulose. In the case of the Me₂N4 + FUdR, the cells were mutagenised by stirring for 1 h at 37 °C in TD buffer with 2.42×10^{-3} M ethylmethylsulphonate (Kodak), washed and grown at sub-confluent density for 2 d in liquid at 38.5 °C before methylcellulose selection. These cells were cycled three times through the methylcellulose-FUdR selection. They were grown in liquid for 2 d at 38.5 °C between each cycle. ND, Not determined.

5-fluorodeoxyuridine to enrich for normal revertants (Table 4). Over 90% of the transformed cells are killed in 2 d growth in FUdR-methylcellulose, whereas normal cells are not significantly affected by the FUdR and survive as well as transformed cells survive in methylcellulose in the absence of FUdR. The mutagenised cells were subjected to three cycles of methylcellulose-FUdR selection with 2-d non-selective growth periods in between. At the end, single colonies were randomly isolated from liquid culture, grown and tested for the expression of the normal phenotype at 38.5 °C shown as the ability to clone in liquid but not in soft agar. In several experiments a total of four stable revertants have been isolated. Knowing the number of viable colony formers at each selection step from plating efficiencies and assuming no differential loss of normal revertants during the experimental procedure, the number of revertants induced by EMS in the original population can be estimated to be 4×10^{-6} per treated cell, 1×10^{-5} per survivor. No revertants were observed without EMS treatment. This reversion frequency is within the range observed for the reversion by *N*-nitro-*N*-nitrosoguanidine of temperature-sensitive mutations for growth in the BHK cell line (0.5 – 5×10^{-5} per cell tested)⁴⁶ and for the reversion by activated carcinogens of a well known temperature-sensitive leucyl-tRNA synthetase CHO cell mutant (2.1×10^{-5} to 4.9×10^{-4} temperature-resistant revertants per survivor)⁴⁷.

In summary, with respect to the characteristics analysed thus far, the chemically-induced BHK transformants are similar to many well documented somatic cell mutants. Although a chromosomal location has not yet been established, the most reasonable explanation for these transformants is that they result from a somatic mutation. The alternative hypothesis that they are of epigenetic origin appears unlikely. In other cases where epigenetic mechanisms have been evoked to explain heritable variations in cultured somatic cells the data have been found unconvincing^{6,48}, and in one instance genetic mechanisms have been shown to be able to explain aberrant results sometimes attributed to epigenesis⁴⁹.

Experiments suggestive of epigenetic carcinogenesis, such as the demonstration that markers from malignant teratocarcinoma cells injected into blastocysts subsequently show

up in normal adult tissue⁵, can be explained in other ways—for instance, by differentiation of the totipotent transformed cells to a state insensitive to the original transforming mutation⁵⁰ or by marker rescue through *in vivo* cell fusion⁵¹. Our findings that (1) three different transformed phenotypes can result from a single treatment of one aliquot of recently cloned cells and (2) half of the total induced transformants are temperature limited in the expression of their transformed phenotype are hard to account for by epigenetic mechanisms, whereas they are easily explained by mutagenesis.

The nature of the gene product which is rendered heat- or cold-sensitive in a temperature restricted transformant is not known. The simplest hypothesis is that it is some product necessary for normal, non-malignant growth. It could act directly or through repression of some oncogene, but the low reversion rate makes involvement of an oncogene unlikely. (Work from this laboratory, to be published soon, indicates that the reversion rate is significantly higher in virus-transformed BHK lines where an oncogene is known to function.) The gene product could be involved in viral transformation although the finding that several temperature-limited clones can be transformed by both RNA and DNA tumour viruses with normal frequencies at the temperature where they display the normal phenotype⁵² suggests that this may not be the case.

A single base change missense mutation is the most likely mutagenic event responsible for the transformation of BHK cells. But the high frequencies with which transformation, an apparently recessive trait in rodent and other cells⁵³, is detected in this nearly diploid line suggest that the line is or becomes functionally haploid at the locus or loci involved in transformation. If a transforming mutation does require a somatic crossover or chromosomal rearrangement or segregation for expression, then these events must be remarkably frequent to account for the high frequency of transformation. The possibility that the carcinogen is not inducing a point mutation at all, but rather is increasing the frequency of chromosomal aberrations which permit the expression of pre-existing alleles is very unlikely. Despite an extensive search, no consistent chromosomal changes have been associated with chemically induced transformation in other Syrian hamster cells^{54,55}. Our observations of three

distinct phenotypes coupled with the wide range of agar plating efficiencies which occur within each phenotype argue against this theory in that the number of pre-existing alleles capable of causing transformation would be excessively large.

Thus, although chemical carcinogenesis in these and other mammalian cells is far from being clearly understood, the evidence which is now available in the case of BHK cells is consistent with the hypothesis that a somatic mutation is the primary event by which chemicals transform normal cells into ones capable of malignant growth.

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letters to nature

Direct observation of individual cosmic ray showers

THE detection of the cores and directions of large cosmic ray showers became possible in the late 1950s as a result of the combination of the techniques of density sampling, using an array of particle detectors to find the shower centre of symmetry¹ and fast timing using spaced scintillation counters to fix the shower arrival direction². Previous Cerenkov light measurements in extensive air showers have concentrated on the gross properties of the light flux on the ground using a similar approach to the particle measurements. It has been suggested by Fomin and Khristiansen³ and Efimov *et al.*⁴ that the full-width half maximum (FWHM) of pulses of Cerenkov light may depend on the longitudinal development of the shower, and so on the atomic mass number of the primary. We have gone further and used precise measurements of the detailed shape of the Cerenkov light pulses recorded by several well spread simple detectors to reconstruct accurately an image of the shower in Cerenkov light. This has been done without the usual prerequisite of first determining the shower's axis of symmetry and arrival direction. The data so obtained may be of use in studies of the average shower characteristics, the search for extreme

fluctuations and, we hope, for the labelling of individual showers with an indication of the mass of their primary.

Measurements were made during about one hundred hours of clear sky, moonless night-time during winter 1975–76 with an array of eight Cerenkov light detectors spread over an area of 1 km² at Haverah Park near Harrogate. Each detector comprised a fast photomultiplier with 5 inch diameter photocathode (RCA type 4522) viewing the night sky directly; the pulses were recorded using a cable delay and oscilloscope display system with a 40 MHz bandwidth. The results to be discussed here are based upon a limited sample of showers (primary energy >10¹⁷ eV) in which at least six detectors recorded Cerenkov light pulses at distances 100–600 m from the shower core. The time of occurrence of light at the 10%, 50% and 90% of full pulse height levels was measured in the rising and falling edges of the pulse to an accuracy of ±2–3 ns from the film records.

The likely origin in the atmosphere of the light observed at various times in the pulse has been made clear by computer simulations. For example, the light pulse at 300 m from the core of a 10¹⁷ eV proton initiated shower is shown in Fig. 1 together with the electron cascade development (Protheroe and K.E.T., unpublished observations). This electron cascade is divided into eight sub-showers and the corresponding eight sub-pulses of Cerenkov

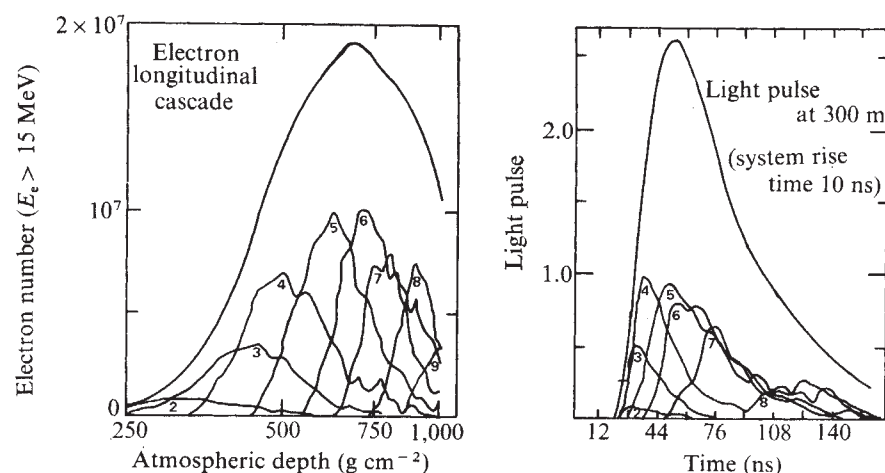


Fig. 1 The longitudinal electron cascade and light pulse shape at 300 m from the core of an average 10^{17} eV proton initiated shower. The light sub-pulses arising from eight sub-showers indicate the origin in this electron cascade of the light in different parts of the light pulse.

light are indicated. It is clear that despite refractive index effects, the earliest observed light originates high in the atmosphere and the pulse shape is clearly a direct measure of the cascade development.

On the assumption that the light at a certain point in each pulse originated from a point in the atmosphere, least square fits of spheres to the sets of times at which the 10% full height was achieved were attempted. These times were found to be very well represented by a sphere (r.m.s. deviation of 3 ns from fronts of radius approximately 7 km (20,000 ns)) and the coordinates of the origin were determined to an accuracy of about 100 m.

The fitting procedure was repeated for the times of the light at the 50 and 90% levels in the rising and falling edges and the origin heights of typical near vertical showers are shown in Fig. 2, after conversion to atmospheric depths in g cm^{-2} . These data show the development of two individual showers in Cerenkov radiation through the atmosphere.

The average measured development of the image of showers of energy about 3×10^{17} eV in Cerenkov light is shown in Fig. 3 together with directly comparable simulation data. The computer simulations were based upon a detailed treatment of air Cerenkov light seen by detectors of the type and bandwidth used in our experiment according to an air shower model incorporating contemporary hadron physics (R. J. Protheroe, G. J. Smith and K.E.T., unpublished). The sensitivity of this technique is clearly shown by the fact that the data of Fig. 3 are based upon showers recorded with zenith angles up to 40° which have very different measured heights of origin (km) but similar depths of origin (g cm^{-2}).

There is no requirement in our analysis of the arrival direction for the centre of symmetry (the core) of the shower, since the spherical light fronts are deduced

directly from the measured pulse shapes and give unambiguously the coordinates of the origin of the light. However, the path traced through these points of origin of light in an individual shower corresponds to the trajectory of the shower core through the atmosphere and provides an accurate measure of the arrival direction (typical errors in angle of arrival are $< 0.1^\circ$). Furthermore, the intersection of this trajectory with the ground plane gives the core impact location (which forms the basis of conventional analyses). Agreement between this impact point and the conventional core location from particle density or Cerenkov light detector arrays is better than

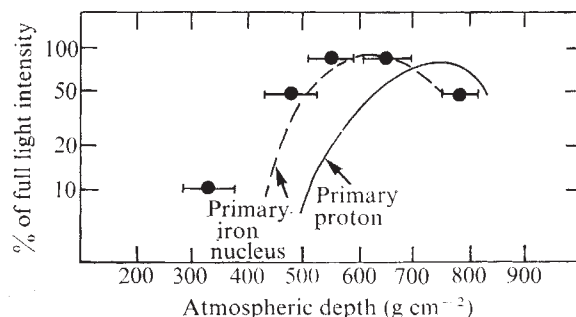
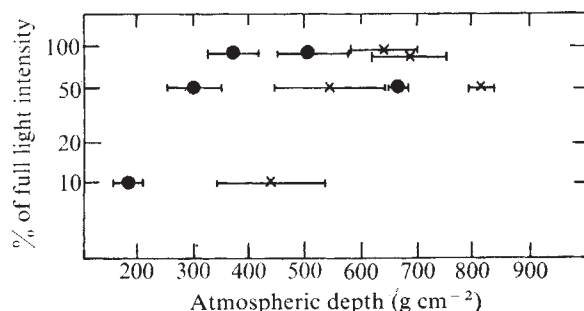


Fig. 3 The average Cerenkov light image of the cascade development of showers (primary energy about 2×10^{17} eV). The broken and solid lines indicate the computed average Cerenkov images for iron nucleus and proton induced showers of comparable energy derived using a shower model incorporating scaling.

Fig. 2 Cerenkov light images of the cascade development through the atmosphere of two showers (primary energy about 10^{17} eV).



50 m in well measured showers. The information available from this technique is summarised schematically in Fig. 4.

It is now possible to rank showers in primary energy in the conventional way on the basis of a photon density at a prescribed distance from the core (simulations indicate a preferred distance of 200–300 m). However, we are also able to rank showers in primary energy from Cerenkov light data without the requirement to know the core position at ground level. This arises since each Cerenkov light detector measures the total light pulse area (which depends strongly upon primary energy and less so upon the individual cascade development, distance from the core of the detector and zenith angle and so on), and the pulse FWHM (which is nearly independent of primary energy and cascade development and varies only with core distance and zenith angle). We regress the values of the pulse area and their corresponding values of the FWHM and obtain that value of the area corresponding to a FWHM of 40 ns. (This corresponds in a conventional

analysis to the pulse area at a core distance of about 300 m.) There is good agreement between this quantity and the conventional measure of the primary energy—the optical density at a distance of 200 m or the Haverah Park ground parameter $\rho(500)_{VE}$ measured by the University of Leeds group—but it emerges without any of the usual core fitting analysis procedures.

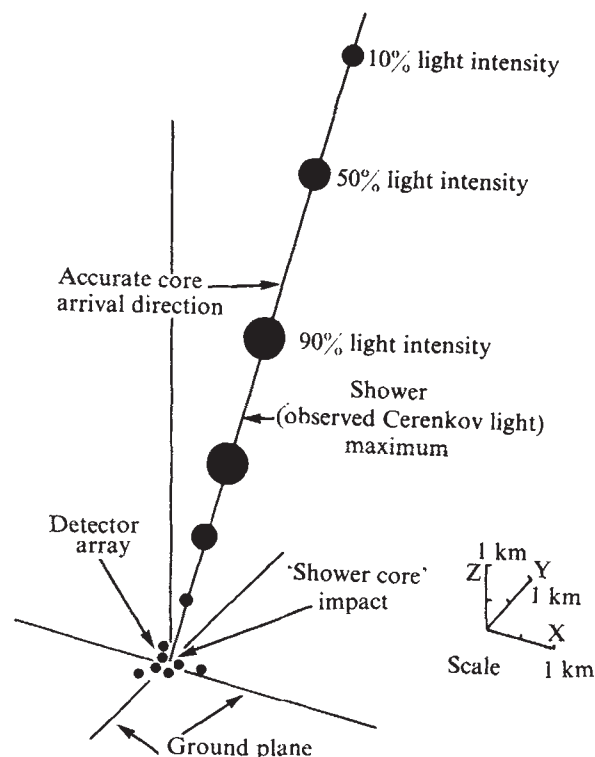


Fig. 4 A Summary of the information available on individual showers from this interpretation.

The main source of error in our present measurement which was made with preliminary equipment is in the time of the beginning of each pulse (the 10% of full light level) relative to the other pulses (r.m.s. error=3.7 ns corresponding to uncertainties of about 100 g cm^{-2} in the initiation depths); the individual pulse shapes are internally well measured (r.m.s. error=1.4 ns) and so the shapes of the inferred shower cascades are at present more reliable than the point of initiation of the shower.

We suggest that a simple, novel and economic method has been demonstrated based upon Cerenkov light measurements for the accurate determination of arrival direction, the estimation of primary energy and, the most important of all, direct observation of the cascade development in individual showers. This method does not depend upon the usual approach to shower analysis and does not require an accurate centre of symmetry (shower core) be determined from ground level data. We consider that measurements of the type described here, employing our improved equipment at locations enjoying long periods of clear sky conditions, may contribute substantially to attempts to measure the atomic mass number of the primary cosmic rays.

We are grateful for the assistance in the operation of the array and the analysis of the data given by Mr R. T. Hammond and Mr D. W. Wellby. The development of this technique was assisted by the availability of the analysis of the Haverah Park particle detector array data

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Possibility of using ^{81}Kr to detect solar neutrinos

THE state of the experiment designed to detect solar neutrinos by the reaction $\nu + ^{37}\text{Cl} \rightarrow ^{37}\text{Ar} (T_{1/2} = 35\text{d}) + e^-$ (threshold = 814 keV) has been summarised by Bahcall and Davis¹, who report essentially a null result for the average of many runs over the past few years. The experiment is sensitive only to a tiny fraction of the total flux, that is to neutrinos arising from the ^8B decay (Table 1) and it is therefore essential to have another experiment which responds to the lower energy, more abundant neutrinos if the fundamental ideas about the mechanisms by which the Sun produces its energy are to be tested. There seem to be very few^{1,2} experiments with this property and an interesting alternative approach has been suggested by Freedman *et al.*³. They hope to obtain a value of the average flux during the past few Myr by measuring the ^{205}Pb produced in old, thallium-rich minerals in the reaction $\nu + ^{205}\text{Tl} \rightarrow ^{205}\text{Pb} (T_{1/2} = 1.6 \times 10^7 \text{ y}) + e^-$ (threshold = 46 keV), which is sensitive to all parts of the neutrino-producing chain. The purpose of this communication is to investigate the feasibility of detecting solar neutrinos through the reaction $\nu + ^{81}\text{Br} \rightarrow ^{81}\text{Kr} (T_{1/2} = 2.1 \times 10^5 \text{ y}) + e^-$ (threshold = 490 keV), which turns out to be sensitive mostly to the ^7Be neutrinos, the second most abundant in the chain.

The requirements for any such "geological" experiment are that the interacting material has been sufficiently deeply buried that the neutrino effect is not swamped by cosmic-ray interactions, that the cover has been in place long enough for any previously produced ^{81}Kr to decay, that the cross-section for neutrino interactions be calculable and that there is sufficient material available. Bromine tends to be a trace element in nature and the chances of obtaining sufficient quantities (estimated at a few hundred kg) of a Br-rich mineral such as AgBr from a great enough depth seem to be low. Certain salt deposits, however, offer a possibility. These are old (Permian, 240 Myr), can be deep (> 1,000 m) and the later crystallisations of such evaporate deposits tend to concentrate Br relative to Cl. For example, in carnallite ($\text{KCl MgCl}_2 \cdot 6\text{H}_2\text{O}$) the ratio of Br:Cl can be 0.01:1 by weight⁴. This mineral is found in large quantities in the Stassfurt deposits and the amount required for a viable experiment can be estimated as follows.

With the above Br:Cl concentration there will be 1.42×10^{19} ^{81}Br atoms per g of carnallite and at saturation (that is after a few

Table 1 Values of the cross-section and interaction rate for the reaction $\nu + ^{81}\text{Br} \rightarrow ^{81}\text{Kr} + e^-$ calculated as described in the text

Reaction	E_ν (MeV)	ϕ ($\text{cm}^{-2} \text{s}^{-1}$)	σ (10^{-45} cm^2)	$\sigma\phi$ (SNU)
$p + p \rightarrow d + e^+ + \nu$	0-0.42	6.1×10^{10}	0	0
$p + e^- \rightarrow p + \nu$	1.44	1.5×10^8	3.02	0.45
$^7\text{Be} + e^- \rightarrow ^7\text{Li} + \nu$	0.86	3.4×10^9	1.06	3.60
$^{13}\text{N} \rightarrow ^{13}\text{C} + e^+ + \nu$	0-1.2	2.6×10^8	0.74	0.19
$^{15}\text{O} \rightarrow ^{15}\text{N} + e^+ + \nu$	0-1.74	1.8×10^8	1.50	0.27
$^8\text{B} \rightarrow ^8\text{Be}^* + e^+ + \nu$	0-14	3.2×10^8	61	0.20

Myr) the number of ^{81}Kr atoms per g will be $1.36 \times 10^{32} \sigma \phi$, where σ is the neutrino interaction cross-section and ϕ the neutrino flux. $\sigma \phi$ is conventionally quoted in solar neutrino units (SNU) where 1 SNU = 10^{-36} interactions per nucleus per s. The flux can be calculated from solar models⁵ and the cross-section obtained from the relations given by Bahcall⁶. Thus for a neutrino-induced reaction which produces an electron in a continuum state of total energy W and momentum p , and in which the neutrino is monoenergetic,

$$\sigma = \frac{2\pi^2 \ln 2 p W F(Z, W)}{ft} \left(\frac{h^3}{m^5 c^7} \right)$$

where $F(Z, W)$ is a tabulated function giving the effect of the nuclear coulomb field on the electron, ft is the comparative lifetime of the reverse, electron capture decay and m is the electron mass.

The ^{81}Kr 7+2 ground state electron capture decay to the 3-2 ground state of ^{81}Br ($Q_{e.c.} = 300$ keV) has $\log ft = 11.6$ and the reverse, neutrino-induced reaction has a very small cross-section. There is, however, a 1-2 isomeric state ($T_{1/2} = 13$ s) of ^{81}Kr at 190 keV excitation and this is the state which would be populated by the neutrino reaction. The electron capture branch of this state has not, unfortunately, been observed (the branching relative to γ emission can be roughly estimated at one in 10^5) and it is necessary to estimate the ft value from the systematics of similar decays in this region, which is an awkward part of the periodic table as far as nuclear theory is concerned because there are several nucleons outside closed shells and a complicated structure of excited states. The following $\log ft$ values emerge: ^{79}As (3-2, ground) $\rightarrow$ ^{79}Se (1-2, isomer): 5.3; ^{81}As (3-2, ground) $\rightarrow$ ^{81}Se (1-2, ground): 5.2; ^{83}Rb (3-2, ground) $\rightarrow$ ^{83}Kr (1-2, isomer): 5.1; ^{83}Br (3-2, ground) $\rightarrow$ ^{83}Kr (1-2, isomer): 5.0. We adopt a value of $\log ft = 5.3$ in the calculation and thus obtain $\sigma = 0.5 \times 10^{-45} W(\text{MeV}) p(\text{MeV}/c) F(Z, W) \text{cm}^2$. (The restriction to 3/2 $\rightarrow$ 1/2 transitions ensures that there are no spin-dependent statistical factors arising from considerations of detailed balance.) The various neutrino-producing reactions are shown in Table 1 with the fluxes (unpublished data of M. S. Freedman *et al.*) and cross-sections calculated from the above formula. In the case of the reactions producing a spectrum of neutrino energies, the average value of the continuous spectrum was used in the calculation. This does not introduce much error because most of the interactions are with the monoenergetic ^7Be neutrinos.

The total interaction rate of 4.7 SNU would yield 6.4×10^{-4} atoms of ^{81}Kr per g of carnallite at saturation. Thus a technically feasible experiment might use 100 tonnes of carnallite dissolved in water (giving 1.5×10^5 l of solution, similar in quantity to the 4×10^5 l of C_2Cl_4 used in the Davis experiment) with the 6.4×10^4 atoms of ^{81}Kr being extracted by methods similar to those used by Davis¹. This number of atoms corresponds to a volume of 2.4×10^{-15} ml of ^{81}Kr at STP, which is about the limit of detection by mass spectrometry in favourable cases⁷. Whether the present case is favourable or not depends largely on the amount of atmospheric Kr which was dissolved in the sea at the time the salt deposits were being laid down and what happened to it thereafter. The Kr content of evaporites does not seem to have been measured but there are some results for Ar (ref. 8) and if these are relevant to Kr it would seem that interference from the scattering tail of ^{82}Kr could be the main problem in the mass spectrometry, for the atom ratio of $^{81}\text{Kr} : ^{82}\text{Kr}$ might be as low as 1:10¹² (personal communication from C. M. Stevens). There is also the question of whether or not the ^{81}Kr produced in the atmosphere by cosmic radiation might be introduced into the mineral. The possibility of escape of Kr during any recrystallisation could be estimated by performing a K-Ar age determination on the mineral and assuming that Kr and Ar behave in a similar way.

To establish whether or not this experiment is possible it is necessary to have a measurement of the ^{82}Kr content of

carnallite, some more information about the availability of suitable quantities of Br and either a better theoretical estimate of the ft value or a measurement of the decay branching. The first measurement is presumably straightforward and would determine whether the idea of carnallite is worth pursuing. If it is not, then the experiment could still be done if Br were available in some other form in which the problem of ^{82}Kr was not so severe. The best hope of an improved ft value probably lies in a search for the electron capture branch of the isomeric decay, using the best resolution Si (Li) detector available to look for Br K X-rays in the presence of a very much greater number ($\sim \times 10^5$) of Kr K X-rays, produced after internal conversion of the 190-keV γ ray. It might turn out, on the basis of the results of the investigations suggested above, that the experiment is impossible—even if all other considerations were favourable, for example, a $\log ft > 6$ would probably make it so. If the solar neutrino problem is to be solved, however, it will probably be necessary to consider any possibility of a viable experiment, no matter how impractical it might seem at first. There are no easy solar neutrino experiments and, almost certainly, they will all be conducted at the outer limits of the techniques involved.

I thank Drs M. S. Freedman and C. M. Stevens, of the Argonne National Laboratory, for arousing my interest in this matter and for helpful correspondence. I thank the staff of the Isotope Geology Unit at SURRC for discussions.

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Prediction of coronal structure of the solar eclipse of October 23, 1976

PREDICTION of solar eclipse coronal structures began in 1968¹ as an offshoot from suggestions by Chapman² and Gold³ that the magnetic field in the vicinity of the Sun could be calculated. A degree of success was achieved with these early attempts^{1,4–6}. Cowling⁷ states that “a sketch showing the corona observed at the eclipse . . . agrees well with the prediction; had Schatten drawn his streamers more nearly radial, the agreement would have been almost perfect”.

This disagreement in the direction of the streamers in the outer corona with those observed arose from the early model's^{8,9} inability to calculate field directions beyond where the solar wind carried out the extended solar magnetic field, approximately one solar radius above the photosphere. A new “current sheet” model¹⁰ has been developed which allows precisely this calculation to be performed. The mathematics and physics of this model are rather complex. Basically, however, the model utilises a current-free inner corona ($1 R_{\odot} < r \leq 1.6 R_{\odot}$), with fields calculated from Legendre polynomials using potential theory fitted to observations of the photospheric line-of-sight magnetic field taken at the Hale Observatories at Mount Wilson. In the outer corona ($r > 1.6 R_{\odot}$), thin, force-free current sheets “open” the magnetic field to the solar wind. They allow the magnetic stress tensor to be balanced everywhere in the outer corona and represent a best Legendre polynomial fit to the previously calculated field at 1.6 solar radii. Force-free current sheets have been shown¹⁰ to resemble closely calculations of isothermal coronas for dipole solar fields¹¹.

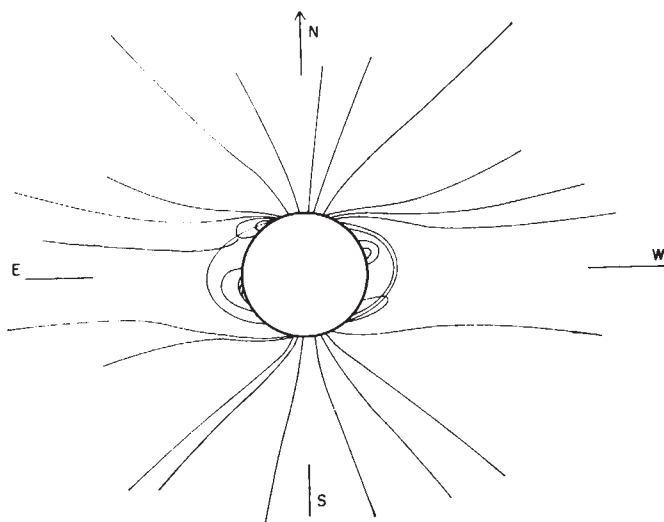


Fig. 1 Sketch of the corona based upon the computer drawn map of the coronal field patterns. The brightest feature of the corona should be the two large equatorial streamers.

The use of force-free current sheets is supported theoretically by the relative weakness of particle pressure compared with magnetic stresses in the outer corona below 20 solar radii⁸.

The photospheric magnetic field data utilised in predicting the eclipse were obtained during Carrington solar rotation 1646. This solar rotation runs from September 13.63 to October 10.90. Thus the east limb data (longitude 104°) are approximately 20 d old on October 23, whereas the west limb data (longitude 284°) are approximately 34 d old on the day of the solar eclipse. Due to the low level of solar activity at present it is thought unlikely that the corona will be seriously disrupted before the eclipse by a new large solar active region.

Before the data could be utilised in the Legendre fit, a considerable amount of preprocessing was necessary at the Hale Observatories. Thus the west limb data are one rotation older at eclipse time than the data during the predictions made in 1968 and 1970. This disadvantage is offset by the considerable advantage gained from having full coverage at all latitudes (except for five degrees near the southern pole of the Sun which are continually tilted away from the Earth at this time). In 1968 and 1970, data equator-ward of 40° latitude were utilised plus mean north and south polar field values. With the digitisation of the data, full polar coverage is now possible.

A computer drawn plot made on October 18 showed the field line structure predicted for the time of the solar eclipse. Figure 1 is a drawing based upon this plot outlining the main features. As can be seen, a very dipolar coronal field results. One huge equatorial streamer is predicted for both the east and west limbs of the Sun. This is due to the lack of very strong active regions near either limb of the Sun. However, nested coronal arches may be seen within this equatorial streamer, below 0.6 solar radii above the limb. Many small arches at latitudes below 60° may be seen on either limb. They are so numerous, they may obscure each other. In particular, on the west limb low arches between 42° and 48° North should appear and on the east limb, low arches near 50° North. In addition, a new active region on the west limb should make its presence known by low arches, and possibly a streamer, near south 26° latitude. However, the main feature of this eclipse should be the two large bright streamers marking the solar equator, with polar plumes in a characteristic dipole fashion.

This total solar eclipse, weather permitting, will be visible over the Indian Ocean, south Australia, and the south Pacific, north of New Zealand. The largest city where this eclipse may be seen will be Melbourne. A number of observers will be there and it is hoped a high resolution photograph with a radial

density filter will allow much of the structure to be discerned for later comparison.

I thank Drs Robert Howard and John Adkins of the Hale Observatories for making available their photospheric magnetic field data. This research was supported by the National Science Foundation, Atmospheric Research Section. I thank Mr Mark Bernstein for his help in handling the Unicol computer which performed the calculations. A copy of the program which performed these calculations can be obtained from the National Space Science Data Center Code 601 GSFC, Greenbelt, Maryland 20771 by asking for the Quiet Sun Magnetic Field Program.

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Another correlated X-ray-radio transition in Cygnus X-1

IN this letter we report that in February 1976, the X-ray and radio emission from Cyg X-1 underwent a simultaneous transition, similar to that observed in March 1971 when the source was first detected at radio frequencies.

The Cyg X-1 region was observed with the Westerbork synthesis radio telescope at 1,415 MHz on four occasions in February–March 1976. Each observation lasted for twelve hours, resulting in an r.m.s. noise of 0.4 mJy. Comparison of the four measurements showed no significant changes in the flux densities of the sources in the field, except for Cyg X-1. The results are shown in Table 1 and Fig. 1.

On February 13, the Cyg X-1 radio source was at a level considerably lower than the lowest level observed since the well known March 1971 transition^{1–5}. Between February 13 and March 6, its intensity rose sharply, peaked, and apparently levelled out close to its usual value of 15 mJy. This behaviour is remarkably similar to that seen in 1971.

Also as in 1971⁷, the dramatic increase in radio intensity was accompanied by a decrease in X-ray intensity by about the same factor⁸. This X-ray transition is shown schematically in Fig. 1. Before the transition, the source had been in its “high” X-ray state since November 1975. It was also in that state for a period of months before the March 1971 transition⁷, and apart from these two occasions, no other instances of such a persistently high emission level have ever been recorded for it⁸.

Between March 1971 and November 1975, the source was in its “low” X-ray state and its radio emission was remarkably constant. The only exception was during May 1975^{5,6}.

Table 1 1,415 MHz flux densities of Cyg X-1

Universal time 1976	S_{1415} (mJy*)
February 13 ^d 03 ^h 59 ^m –13 ^d 16 ^h 01 ^m	5.0 ± 0.6
February 22 03 24 –22 15 26	18.5 ± 1.0
February 27 03 04 –27 15 06	23.1 ± 1.2
March 06 02 33 –06 14 35	18.3 ± 1.0

*1 mJy = 10^{-26} W m⁻² Hz⁻¹

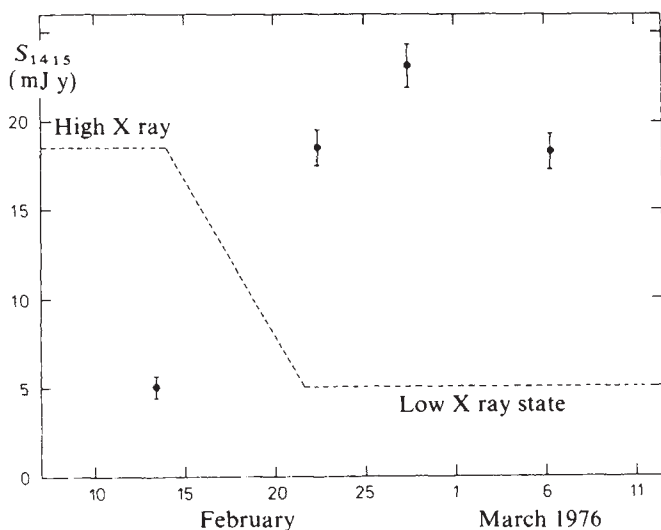


Fig. 1 The radio and X-ray behaviour of Cyg X-1 during February–March 1976. The radio data are from Table 1. The dashed line gives a schematic representation of the Ariel V X-ray data taken from Holt *et al.*⁸.

At that time, the X-ray and radio emission both increased, distinguishing that short lived “transient event” from the “transition” reported here.

There are several possible explanations for the anticorrelation observed in the X-ray and radio behaviour of Cyg X-1 during transitions. First, heightened X-ray emission could be produced by a hot gas which eventually envelops the radio emitting region, blanketing it out by free-free absorption. When the gas expands sufficiently, the cut-off frequency would become lower and the radio source would reappear in its normal state. Second, the event responsible for the enhanced X radiation might either interrupt the supply of relativistic electrons to a non-thermal radio source or result in such an increased radiation density that inverse Compton losses would extinguish the source. A cut-off of the additional X-ray supply would then again result in a return of the radio source to its normal state. To decide between these and other alternatives, it is crucially important to monitor the radio spectrum of Cyg X-1 throughout future transitions.

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New test of the cosmological nature of QSO redshifts

IN spite of extensive observations the nature of the redshift of a quasistellar object (QSO) remains a controversial issue¹. The redshift–magnitude relation, QSO–galaxy associations, time variability faster than light motion and several other criteria are being used to argue in favour of or against the cosmological nature of the QSO redshift. It is therefore desirable not only to improve the statistics of the existing data but also to think of new tests to clarify this important issue. Recent improvements^{2,3} in the spectrophotometry of QSOs enable us to propose a potential test of the cosmological hypothesis in the following manner.

Consider a plot of the emission line width (for example, the full width at half maximum intensity, FWHM) of a specified spectral line against the redshift of a QSO. Assuming that all QSOs are intrinsically identical, and are at cosmological distances corresponding to their redshifts, the FWHM of a typical line of rest wavelength λ_0 in a QSO of redshift z is given by

$$w = w_0(1+z) \quad (1)$$

where w_0 is the FWHM in the rest frame of the QSO. A plot of w against $1+z$ should therefore be a straight line through the origin. The test involves plotting w against $1+z$ for a large number of QSOs, using the same spectral line for all of them.

In a hypothesis where the redshift is largely of gravitational origin there is no simple model-independent relation like equation (1). For example, in the model of the type considered by Greenstein and Schmidt⁴, the line width arises from the gravitational broadening at the surface and the relation between w and z is given by

$$w = \frac{R_g}{2R} \lambda_0 (1+z)^3 \frac{\Delta R}{R} \quad (2)$$

where R_g is the gravitational radius of the object, $4\pi R^2$ is the surface area of the object and ΔR the thickness of the emitting region in the Schwarzschild coordinates. Both R and ΔR depend upon the model. For the Hoyle–Fowler type models with central emission line redshifts⁵ even more complicated forms than equation (2) arise.

For the local Doppler shift explanation of the type advanced by Terrell⁶ the relation (1) will hold. Thus a tight straight-line plot will be a positive test of the cosmological or Doppler hypothesis. In practice, however, the test is not so easy to apply and we discuss briefly some of the difficulties and how they could be resolved.

First, the measurements of FWHM have to be made very accurately. With the image tube scanner technique² it is possible to have resolutions better than 7 Å in FWHM. Since the line widths are at least 20 Å in most cases it now seems possible for at least a start to be made. It is of course necessary to choose the same spectral line for all QSOs in any given sample. Thus Ly α may be chosen for high redshift QSOs ($z \gtrsim 1$) and the MgII line for low redshift QSOs ($z \lesssim 1$). The CIV line can also serve a good line for a large number of QSOs of moderately high redshifts.

A preliminary ($w, 1+z$) plot for a sample of southern sources^{7,8} reveals a large scatter (see Fig. 1) for the MgII line of low redshift QSOs. A similar plot for the CIV line in a number of high redshift QSOs based on the data given by J. Baldwin (personal communication) also shows no linear relationship. The scatter may of course be due to variation in w_0 from QSO to QSO, and this illustrates another difficulty of arriving at a decisive conclusion. However, intrinsic line widths probably do not vary by more than a factor of 10 and the scatter could be reduced by properly selected criteria, for example the nature of radio spectra⁹. To arrive at a suitable criterion it is necessary to have a better understanding of the causes of emission lines

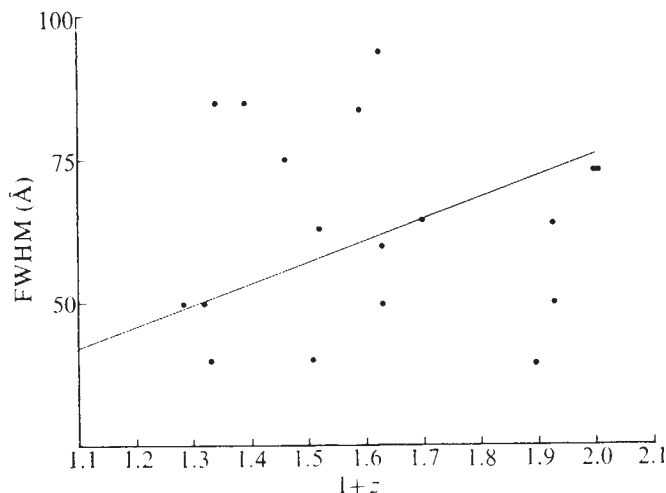


Fig. 1 Plot of FWHM against $1+z$ for MgII line for 18 southern sources. The scatter about the line of least square fit to relation (1) shows how at present no linear trend is apparent from the data.

in QSOs from theoretical as well as from empirical approaches based on observations¹⁰. The situation is no worse than in the $m-z$ relation for QSOs, where the scatter is supposed to arise (if the cosmological hypothesis is right) from the variation in the intrinsic luminosity by several magnitudes. On the other hand the proposed test is free of the added uncertainty of not knowing the deceleration parameter q_0 in the $m-z$ relation.

It is possible to apply this test to emission line galaxies such as Seyferts, but the smallness of the redshift makes the variation of w with z insensitive according to equation (1). For QSOs, with their large redshifts, this test appears better suited. With the redshifts and extensive optical information on more than 570 QSOs available (Burbidge, Crowne and Smith, preprint) it may be worthwhile attempting this test with better measurements of line widths which should become possible in the future.

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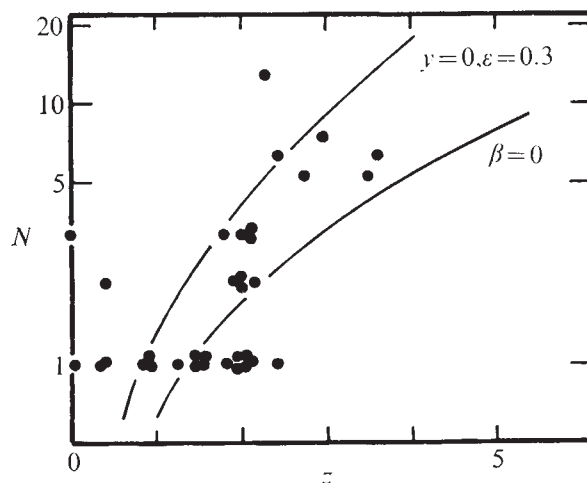
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However, the small internal velocity dispersions inferred for the clouds from the absorption line widths ($\sim 50 \text{ km s}^{-1}$) pose serious problems to this model⁹ and we consider here the second interpretation. The interception cross section of intervening galaxies for QSO photons has hitherto been calculated¹⁰ on the assumption that the galaxies do not evolve. The data so far available (Fig. 1), although probably contaminated by selection effects¹, seem to indicate that the number of absorption redshift systems increases more rapidly as a function of the emission line redshift than can be accounted for without such evolution. The possibility has been considered^{7,8} that the intervening clouds are collapsing protogalaxies, but we think it likely that most galaxies form¹¹ before $z=5$ and, instead, attribute the rapid increase in the number of redshift systems to tidal interaction of galaxies at earlier epochs. Because galaxies were closer together in the past, chance collisions were more frequent and the effects of these interactions were more pronounced.

The effects of an encounter between a galaxy and a neighbour are proportional to the tidal force and to the duration of the encounter. This force is inversely proportional to the cube of the distance r between the galaxies; the duration of the collision is proportional to r/v , where v is the relative velocity of the galaxies (since we intend to calculate the probability of a tidally distorting collision as a function of z only, the vectorial properties of the collision are irrelevant). Thus, the amount of disruption that a galaxy suffers in an encounter is proportional to $r^{-2}v^{-1}$. The expectation value of this quantity can be calculated if we know the probability of finding, near any given galaxy, another one with distance between r and $r+dr$ and with velocity between v and $v+dv$. In other words, we must know how the phase space spanned by r and v is occupied and then convolve $r^{-2}v^{-1}$ with the density distribution in phase space.

The average distribution is found from the cosmological model used to describe the large-scale evolution of the Universe, but in addition the effects of clustering must be considered. The empirical evidence on the pair correlation of galaxies¹² is unfortunately insufficient for our purposes, first because the dependence of the correlation on redshift is unknown, second because the velocity correlation is unknown. We are currently working on the results of an N -body model of the evolution of clustering to determine the density distribution in phase space and here anticipate the results by assuming the Keplerian approximation $v \propto r^{-1/2}$ and by assuming that the clustering at

Fig. 1 Number N of absorption redshift systems against the emission redshift of the background QSO. Dots: observations¹; drawn lines: predictions for a universe with $q_0 = 0$. Lower curve: no tidal distortion. Upper curve: tidal distortion parameter $\epsilon = 0.3$, clustering parameter $\gamma = 0$. Because of incompleteness of the sample, N has not been expressed as a fraction of the total number of sources observed. Therefore, the observed points may be fitted to the curves by applying an unknown multiplication factor to N , which in the diagram corresponds to vertical displacement.



Interacting galaxies and QSO absorption lines

THE spectra of quasi-stellar objects are observed to have absorption lines with redshifts mostly below the redshift of the emission lines¹. The absorbing clouds are thought to be either near the QSO¹⁻⁵ or at cosmological distances from it⁶⁻⁸. On the first interpretation, the clouds are supposed to be accelerated to high velocities ($\lesssim 0.5c$ away from the QSO) by radiation pressure.

redshift z can be described by the usual linear perturbation of the cosmic number density,

$$n = n_0(1+z)^3[1 + (1+y)/(1+z)] \quad (1)$$

where y is the redshift at which the density perturbation reaches unity.

Under these assumptions, the expectation value of the factor β by which the projected area of a galaxy increases due to an encounter is $\beta \propto r^{-2}v^{-1} \propto r^{-3/2}$, and because $r \propto n^{-1/3}$ we obtain

$$\beta = \varepsilon(n(z)/n_0)^{1/2} = \varepsilon(1+z)(2+y+z)^{1/2} \quad (2)$$

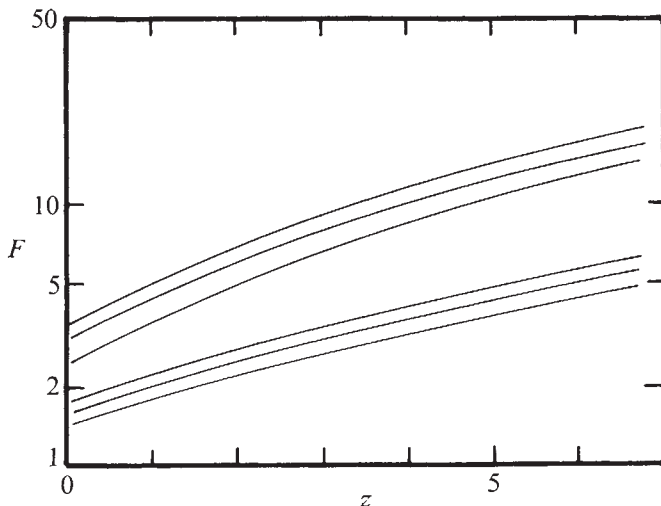
The parameter ε is estimated by noting that numerical simulations¹³ give at least a fivefold area increase for a collision with a periastris of a few galactic radii. Thus, if the mean distance between galaxies with a mass of 10^{41} kg is 1.5 Mpc, one has $\varepsilon \approx 5(0.1/1.5)^{3/2} = 0.07$. Observations of the interacting system M81/M82 show¹⁴ a hydrogen envelope having a projected surface area of about 10 times the combined Holmberg areas of the individual galaxies. This suggests $\varepsilon \approx 0.2$, but the collision may be atypical. With equation (2), the probability P that the line of sight from us to a QSO with redshift z cuts a galaxy or a tidal extension is given by

$$P \propto \int_0^z (1+z')(1+\beta(z'))dz' \\ = \left[\frac{1}{2}(1+z')^2 + \varepsilon^2 \frac{2}{3}(2+y+z')^3 \right] \left\{ (1+z')^2 - \frac{4}{3}(1+z')(2+y+z') + \frac{8}{35}(2+y+z')^2 \right\} \Big|_0^z \quad (3)$$

It has been assumed that $q_0 = 0$, which avoids complicated functions in (3) and is for the present purposes sufficiently near the value $q_0 = 0.05$ that corresponds to the mean cosmic density implied above.

In Fig. 1 we have drawn the function (3) for $\varepsilon = 0$ (see ref. 10) and in Fig. 2 the factor with which this must be multiplied to obtain the solution for various values of ε and y . This factor equals the relative proportion of lines due to bridges and tails of all absorption line systems. The optical depth¹⁰ of the lines in absorption systems due to a bridge or a tail will be smaller than those due to galaxies proper. The ionisation state of the tidal extensions could be caused by the higher ultraviolet background radiation expected^{7,8} at higher values of z . As appears from the

Fig. 2 Factor F with which the number of intercepting galaxies expected for $q_0 = 0$ (see text) must be multiplied in order to obtain the number expected when tidal interaction is taken into account. Upper triple: tidal distortion parameter $\varepsilon = 1$, lower triple $\varepsilon = 0.3$. Of each triple, the upper curve has clustering parameter $y = 4$, middle curve $y = 2$, lower curve $y = 0$.



figure, tidal extensions could contribute significantly to the interception cross section of galaxies at large redshifts.

Predictions of our model are (1) because the probability of tidal deformation increases with z , the fraction of QSOs with shallow absorption lines also increases with z , (2) the abundances of the absorbing atoms are typical for the interstellar medium: (3) absorption line splitting of the order of a few hundred kilometers per second (that is, the velocity at periastris of slightly hyperbolic orbits) is possible, but the splitting should not be 'magic'. Observational tests on the basis of existing data are not yet possible for (1), confirm^{1,2} (2) and are inconclusive^{1,5} for (3). We feel that the third one is the acid test: it is, of course, a key prediction of our model that the absorption line splitting will not be constrained to certain 'magical' intervals in frequency.

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Theoretical maximum for energy from direct and diffuse sunlight

SCHEMES for the conversion of sunlight to useful (electrical, mechanical, chemical) energy all make use of the high spectral temperature of solar radiation relative to the terrestrial ambience. Some schemes, but not others, also make use of the high directivity of the solar flux. For example, focusing mirror arrays require direct sunlight, while photovoltaic devices are indifferent to the directness or diffuseness of light of a given intensity. In biological systems, photosynthesis evidently makes little use of sunlight directivity, since it is not observed to depend strongly on plant orientation on angular scales as small as half a degree (the size of the Sun); on the other hand, Kevan¹ reports some heliotropic, arctic flowers whose corollas are nearly paraboloidal and focus direct (but not diffuse) radiation on the sporophylls.

The maximum thermodynamic efficiency permitted for extracting energy from direct sunlight must be higher than that permitted for diffuse sunlight of the same intensity, since loss of directivity is evidently an irreversible process. But what are the numerical values of these two maximum efficiencies? Are they different enough to justify *a priori* a concentration of effort by man or plant on the exploitation of direct solar radiation only? These are the questions to be considered briefly here, somewhat more specifically than can be found in the literature^{2,3}. The answers are in some ways surprising. For example, neither direct nor diffuse light yields the efficiency formula $(T_{\odot} - T_{\oplus})/T_{\odot}$ that

one might naively expect (where $T_{\odot}$ is the equivalent black-body solar temperature, $\sim 5,800$ K, T_e is the ambient temperature on Earth, ~ 300 K).

We take the input thermodynamic system to be a volume sample of the ambient radiation field at the Earth's surface, including fluxes from the Sun, sky and ambient surroundings. Per unit volume suppose that this radiation field has energy E and entropy S . Since a black-body (maximum entropy) distribution has an energy density $a(S/(4/3)a)^{4/3}$, an amount of work equal to $E - a(S/(4/3)a)^{4/3}$ can be extracted isentropically, leaving a black-body distribution of temperature $T_{\star} = (S/(4/3)a)^{1/3}$. (Here a is the usual radiation constant.) Adiabatic expansion or contraction of the unit volume now extracts further work, until the radiation temperature is brought to equilibrium with the ambient temperature T_e . This amount of work is easily computed to be $aT_{\star}^4 + (1/3)aT_e^4 - (4/3)aT_{\star}^3T_e$. The total useful work is thus

$$R = E - ST_e + \frac{1}{3}aT_e^4 = G(T_e) \quad (1)$$

where $G(T_e)$ is the Gibbs free energy⁴ of the original unit volume evaluated relative to the ambient temperature T_e and its external radiation pressure $(1/3)aT_e^4$.

We must now compute S and E for the examples of interest: If $Ndv d\Omega$ is defined to be the volume density of photons in a frequency interval dv and solid angle $d\Omega$, then (taking units with $c = 1$),

$$E = \int N h \nu \, dv d\Omega$$

$$S = \int \left[2\nu^2 \ln \left(1 + \frac{N}{2\nu^2} \right) + N \ln \left(1 + \frac{2\nu^2}{N} \right) \right] dv d\Omega \quad (2)$$

The integration extends over all angles, and from 0 to ∞ in ν . Equation (2) goes back to Planck⁵ and others⁶.

Equations (2) and (1) could now be evaluated numerically using the empirically known radiation field near the Earth. A useful approximation, however, is to idealise the field as the sum of various, possibly diluted, black-body contributions. Such a flux with dilution factor ϵ has $N = 2\epsilon\nu^2/(\exp(h\nu/kT)-1)$, and if it is from solid angle ω , then the integrals (2) can be done by computer and expressed as an accurate numerical approximation:

$$E = aT^4(\omega/4\pi)\epsilon \quad (3)$$

$$S = (4/3)aT^3(\omega/4\pi)\epsilon(0.9652 - 0.2777 \ln \epsilon - (0.0348 + f(\epsilon))\epsilon)$$

where $f(\epsilon)$ is a smooth function which can be neglected for $\epsilon < 0.01$; $f(1) = 0$, $f(0.1) = 0.0114$, $f(0.01) = 0.012$.

Consider now direct sunlight: The solar black-body contribution is undiluted at temperature $T_{\odot}$ and occupies a fractional solid angle $\omega/4\pi = 5.4 \times 10^{-6} \equiv \delta$ (the size of the Sun in the sky). The remaining solid angle is approximately a black-body of temperature T_e , also undiluted. Combining equations (2) and (3) then gives

$$R = \delta a T_{\odot}^4 \left[1 - \frac{4}{3} \frac{T_e}{T_{\odot}} + \frac{1}{3} \frac{T_e^4}{T_{\odot}^4} \right] \quad (4)$$

The term in square brackets is seen to be the optimal efficiency, and it has a numerical value 0.93 for $T_{\odot} = 5,800$ K, $T_e = 300$ K. Next consider diffuse sunlight of intensity identical to the above. Here we set $\omega/4\pi = 1$ for both solar and ambient components, but set ϵ , the dilution factor, to δ for the solar contribution, and $\epsilon = 1 - \delta$ for the ambient contribution. Now

equations (2) and (3) combine to give (for $1 \gg \delta \gg \exp[-T_{\odot}/T_e]$)

$$R = \delta a T_{\odot}^4 \left[1 - \frac{4}{3} \left(0.9652 - 0.2777 \ln \delta \right) \frac{T_e}{T_{\odot}} + O \left(\frac{T_e^4}{T_{\odot}^4} \right) \right] \quad (5)$$

The numerical value of the bracketed efficiency coefficient is here 0.70. Diffuse sunlight, then (or direct sunlight with a conversion scheme which does not make use of its directivity), allows about 25% less conversion of energy. This is not simply the geometrical effect of increased flux on to a normal surface (the radiation being sampled on a volume basis), nor is it the effect of a lower total flux which is the general concomitant of diffuse radiation (since we have equalised the intensities in the above calculation); rather it is a consequence of the fundamentally greater entropy in the diffuse radiation, hence its smaller free energy.

To decide whether this is an important efficiency difference, we can note that even on a cloudless day with the Sun directly overhead, of order 20% of the total solar flux is diffuse, and an average temperate cloud cover raises this fraction towards unity with 60% typical⁷. One concludes that any scheme for using diffuse sunlight at near-maximum efficiency (and direct sunlight therefore at an automatic sacrifice of only $\sim 25\%$), should dominate a scheme which optimises for direct flux (and, for example, with focusing mirrors, sacrifices diffuse radiation almost completely).

In a sense, we were anticipated in this conclusion by the evolutionary experience of natural plants. The advantage of a differently "designed" photosynthesis at the photomolecular level, one which might use the directionality of the solar radiation on angular scale $\sim 0.5^\circ$, does not seem to have been sufficient to have driven terrestrial evolution in this direction. One is left to speculate about whether exobiological evolution under different conditions might be able to find a different chemistry for photosynthesis, one which uses the free energy of directionality in addition to (or instead of!) the free energy of spectral temperature.

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Reduction of visibility by sulphates in photochemical smog

THE relationship between pollutant emissions and the optical haze characteristic of photochemical smog has proved difficult to unravel^{1,2}. It is clear that material produced by reaction in the atmosphere is responsible for much of the deterioration in the optical environment, since the ambient aerosol scatters much more light at a given mass concentration than do the primary aerosols emitted by known sources^{3,4}. Unfortunately, measurement of the scattering contributed by an individual product species is complicated by the fact that most of this secondary material is deposited on existing particles and

cannot be isolated for direct observation of its *in situ* optical properties. In this note I present statistical evidence from measurements in Los Angeles that secondary sulphate compounds scatter visible light more efficiently than do other chemical fractions of the ambient aerosol. My results indicate that sulphur emissions can strongly influence atmospheric visibility even in photochemical smog of the Los Angeles variety, where secondary organics and nitrates contribute most of the pollutant aerosol mass.

Visibility in a polluted atmosphere is determined primarily by the total scattering coefficient (b_{scat}) of the ambient aerosol⁶. As part of the California Aerosol Characterisation Study (ACHEX)⁷, the scattering coefficient, mass concentration, and chemical composition of the Los Angeles aerosol were monitored during selected photochemical smog episodes in the summer of 1973. Of particular interest are data from 28 two-hour sampling periods of low ($\leq 60\%$) relative humidity, when scattering from water absorbed by hygroscopic constituents of the aerosol was minimal^{8,9}. The scattering coefficient averaged $6.4 \times 10^{-4} \text{ m}^{-1}$ over this sample, and showed significant correlation with aerosol mass concentration and sulphate concentration. Ninety per cent of the observed variance in the scattering coefficient is accounted for by the following least-squares fit (NSA: non-sulphate aerosol):

$$b_{\text{scat}} = 7.6 \text{ m}^2 \text{ g}^{-1} [\text{SO}_4^{2-}] + 2.4 \text{ m}^2 \text{ g}^{-1} [\text{NSA}] \pm 1.0 \times 10^{-4} \text{ m}^{-1}$$

In this expression, $[\text{NSA}] = [\text{aerosol}] - 1.3 [\text{SO}_4^{2-}]$ is the calculated concentration of non-sulphate aerosol, the factor 1.3 representing the mass ratio of sulphate compound to sulphate ion.

The value 1.3 used above for the compound/ion (C/I) ratio was chosen as representative of the likely atmospheric mix of $(\text{NH}_4)_2\text{SO}_4$ (C/I = 1.38), $(\text{NH}_4)\text{HSO}_4$ (C/I = 1.20), Na_2SO_4 (C/I = 1.48), NaHSO_4 (C/I = 1.25), and H_2SO_4 (C/I = 1.02), based on the relative concentrations of sulphate, nitrate, ammonium, and sodium found in the aerosol¹⁰. The compound/ion ratio can also be estimated by purely statistical means, and this provides a way to test the experimental data for the possible influence of "hidden" variables statistically associated with sulphate. Multiple linear regression of aerosol mass concentration on $[\text{SO}_4^{2-}]$, $[\text{NO}_3^-]$, and $[\text{C}]$ over all relative humidities gives a value of 1.2 ± 0.2 for the $[\text{SO}_4^{2-}]$ coefficient, which is consistent with the compound/ion ratios of the probable aerosol sulphates identified above. (The $[\text{SO}_4^{2-}]$ coefficient in the corresponding regression of b_{scat} on $[\text{SO}_4^{2-}]$, $[\text{NO}_3^-]$, and $[\text{C}]$ over all relative humidities is $9.8 \text{ m}^2 \text{ g}^{-1}$.) The accuracy of the statistical estimate for the contribution of sulphates to aerosol mass lends credibility to my statistical estimate for the contribution of sulphates to light scattering.

The empirical b_{scat} -aerosol relationship presented here indicates that sulphate compounds scatter much more efficiently at low humidities than the remainder of the aerosol does. During the sampling intervals for which the relationship was derived, sulphate compounds apparently contributed almost a third of the average scattering coefficient, although they contributed only $30\text{--}35 \mu\text{g m}^{-3}$ ($[\text{SO}_4^{2-}] = 25 \mu\text{g m}^{-3}$) towards the average mass concentration of $220 \mu\text{g m}^{-3}$. The relationship was fitted to a limited set of data from a limited geographical area, and should be applied to other situations only with considerable caution. It is worth noting, however, that the reciprocal of the $[\text{SO}_4^{2-}]$ coefficient falls within the range of $[\text{SO}_4^{2-}]/b_{\text{scat}}$ ratios reported by Waggoner *et al.*¹¹ for aerosols dominated by sulphates. This raises the possibility that the optical activity of the sulphate compounds results from basic physical and chemical mechanisms which govern their distribution in the aerosol with respect to particle size, as suggested in a more general context by Friedlander¹². Further measurements will be necessary before this hypothesis can be confirmed or disproved.

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Exsolution in 'stoichiometric' mullite

MULLITE, first identified only fifty years ago, was quickly recognised to be an important industrial mineral. Its chemical composition was determined initially as $3\text{Al}_2\text{O}_3 \cdot 2\text{SiO}_2$ on the basis of two chemical analyses^{1,2} and the observation that when a synthetic mixture of 3:2 composition was fired to temperatures just below the liquidus, no glass or corundum was observed¹. Although more aluminium-rich mullites were found subsequently³, the 3:2 or "stoichiometric"⁴ composition remains the generally accepted one.

In the course of a study of solid solution in mullite a number of occurrences of rocks with coexisting sillimanite and mullite were found⁵. The most obvious way that this could come about would be if reactions (1) or (2) had not gone to completion:



Reaction (1), a common synthetic mullitisation process, takes place at about $1,400^\circ\text{C}$ at 1 atmosphere while thermodynamic calculations^{6,7} indicate that mullite should form according to reaction (2) at about 900°C . Two specimens of

Fig. 1 Mullite with exsolved sillimanite, specimen Al 37y, Thorncliffe, Transvaal, South Africa. *a*, Overfocused optical micrograph of a grain mounted in oil at 45° position under crossed polars. *b*, Electron micrograph showing lamellae of sillimanite (light) in a mullite host.

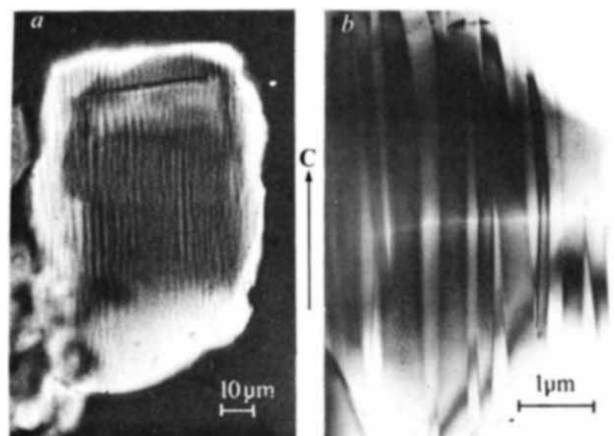


Table 1 Chemical compositions and cell dimensions of the sillimanite and mullite phases

	Electron probe		EMMA-4	
	"Mullite"	Sillimanite	Mullite host	Sillimanite
Al ₂ O ₃	69.7	63.0	74.1	63.7
SiO ₂	28.9	36.1	24.4	35.8
TiO ₂	0.8	0.07	—	—
Fe ₂ O ₃	0.3	0.3	—	—
Cr ₂ O ₃	0.3	0.2	—	—
Total	100.0	99.7	98.5	99.5
Mol % Al ₂ O ₃ + TiO ₂ : Fe ₂ O ₃ + Cr ₂ O ₃	59.0(5)	50.9(5)	64.5(2.0)	51.4(2.0)
Cell dimensions				
		Mullite	Sillimanite	
<i>a</i> (Å)		7.5482(7)	7.4861(9)	
<i>b</i> (Å)		7.6824(15)	7.6827(21)	
<i>c</i> (Å)		2.8876(3)	2.8879(4)	
<i>V</i> (Å ³)		167.45(3)	166.09(4)	

Experimental details for the electron probe and powder X-ray work as in ref. 5. EMMA-4 instrumental conditions are those described by Cliff and Lorimer¹³. Al/Si ratios were recalculated as oxides to 100%, allowing for estimated trace element contents. The results quoted are the average of 10 analyses. Figures in parentheses represent the standard deviation in terms of least units cited for the value to their immediate left.

corundum-sillimanite-mullite xenoliths from different localities within the Critical Zone of the Bushveld Intrusion⁸ were sufficiently coarse grained to afford detailed examination under the polarising microscope. The absence of a silica phase either within mullite grains or at grain boundaries suggests that reaction (2) was the mullite-forming process. Indeed, in specimen Al 37y, sillimanite is found preferentially in contact with (?)relict corundum grains.

The ratio of sillimanite to mullite, estimated optically, in specimens Al 37y and JW II 5 was very much less than that determined by X-ray powder diffractometry⁵. Coupled with the fact that sillimanite, not mullite, is found in the outermost zone of the Thorncliffe xenolith where it reacted *in situ* with an anorthositic host, there seemed a strong possibility that earlier formed mullite in the core of the xenolith may have exsolved sillimanite after settling near the floor of the magma chamber. In thin section individual grains have a distinctly mottled appearance under crossed polars and, when mounted in oil, almost all show evidence of microstructure parallel to the *c* axis of the crystals on a scale of about 0.1 µm both under crossed polars (Fig. 1a) and in plane polarised light. This implies that a second phase with a slightly different refractive index is present; indeed the overall texture is strongly reminiscent of exsolved orthoamphiboles⁹ and materials that have undergone spinodal decomposition but on a very much coarser scale¹⁰.

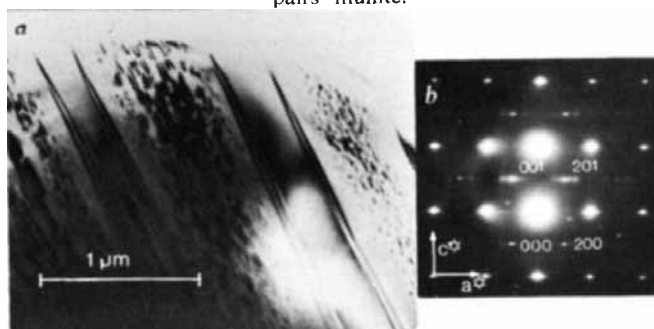
An electron microscope study (AEI EM6G) of ion-thinned crystals from both specimens revealed sinuous lamellae up to 0.2 µm wide and attaining lengths of several tens of microns (Fig. 1b). Diffraction patterns from the lamellae showed the simple 2*c* superlattice reflections characteristic of sillimanite. The host displayed intensity maxima at irrational values of *h*, *k* and *l*, typical of the antiphase domain structure adopted by mullite¹¹. The plane of contact is (100), that of the greatest mismatch between the two unit cells (about 1% from Table 1). Within the mullite phase are tiny platelets (0.1 × 0.012 µm) which become smaller and finally disappear near sillimanite lamellae, leaving a precipitate-free zone 0.05–0.15 µm wide (Fig. 2a). The *a**-*c** diffraction pattern (Fig. 2b) taken from an area 0.5 µm in diameter in the mullite host midway between two sillimanite lamellae shows that the platelets also have the sillimanite structure and are in precisely the same crystallographic orientation as the mullite.

One must conclude that sillimanite exsolves on (100) of mullite during very slow cooling. The wide lamellae are likely to have formed at temperatures as high as 1,100 °C but diffusion of Al (and Si) was sufficiently sluggish for a concentration gradient to be set up in the mullite host, resulting eventually in the exsolution of a second generation of sillimanite. Augite exsolution from orthopyroxene is an almost perfect analogy¹² except that in the case of mullite the tiny platelets within the host seem to be those of the equilibrium phase and are there-

fore not Guinier-Preston zones. A certain amount of disorder does exist, however, in the sillimanite as evidenced by streaking in the diffraction pattern parallel to *a**.

The bulk composition, that of the initial mullite, was found by electron probe microanalysis (beam diameter 2 µm) to be 59.0 mol % Al₂O₃(+TiO₂+Fe₂O₃), only slightly off the 3:2 stoichiometry (Table 1). The quoted sillimanite analysis is that from a few crystals of apparently homogeneous sillimanite in the Thorncliffe specimen and is assumed to be similar to that of the coarsely exsolved lamellae. Two approaches towards finding the compositions of the exsolved phases were adopted. Use of the relationship between the *a* cell dimension and Al content in synthetic and natural mullites with very different thermal histories (W.E.C., unpublished) gave the chemical composition of the mullite lamellae as 61.3 ± 0.3 mol % Al₂O₃. The powder diffraction peaks of sillimanite were slightly broader than those of mullite, resulting either from the disorder mentioned previously or from a small compositional difference between the two scales of lamellae. The *a* parameter, however, was lower than those in sillimanites with similar minor element contents from metamorphic rocks formed at similar pressures while the cell volume was typical of sillimanites with a small amount of excess Al. EMMA-4 analyses (beam diameter 0.1 µm) using *k* values¹³ determined on the Brandywine Springs sillimanite and an analysed mullite, 76398 (ref. 14), are given in Table 1. Thin, precipitate-free mullite lamellae were chosen and for all analyses, great care had to be exercised in determining background levels. EMMA results, in spite of their high estimated errors, confirm the presence of excess aluminium

Fig. 2 Nature of the precipitates within the mullite host. *a*, Electron micrograph showing the second generation of exsolved sillimanite. Contrast fringes at the sillimanite-mullite interfaces arise from inclined planar defects, observed when the beam is not quite perpendicular to (010). *b*, *a**-*c** electron-diffraction pattern from the mullite host, indexed on the *c* = 2.9 Å cell. The sharp superlattice reflections characterise sillimanite; the diffuse pairs mullite.



in the sillimanite and provide a very much more reasonable value for the mullite composition considering the volume relationships shown in Fig. 1b. The cell dimensions, in any case, are likely to be suspect owing to the possibility of coherent or semi-coherent exsolution, as in perthites¹⁵. The actual mechanism of exsolution is most likely to have been by nucleation and growth, in spite of the even distribution of precisely oriented sillimanite lamellae which one might ascribe to a coarsened spinodal. The solute profile inferred for the mullite host is the reverse of that which would arise by spinodal decomposition.

The observation that mullite has exsolved to give a non-stoichiometric sillimanite and another mullite with about 64 mol % Al_2O_3 argues very strongly against the presence of any 3:2 "ordered"¹⁶ compound at high temperature. Slowness of diffusion of Al and Si was the main constraint on the decomposition reaction and instead of nucleating the thermodynamically stable phase, corundum, the host phase preserved the topology of an initially crystallised mullite. The Bushveld specimens represent the closest approach to equilibrium for reverse reaction (2) that is ever likely to be found: makers of mullite refractories need have little cause for concern.

This paper has benefitted greatly from the technical skill and advice of Mr G. Cliff and from discussions with Drs G. W. Lorimer, P. E. Champness and J. D. C. McConnell. Mr E. A. Viljoen kindly provided the Bushveld specimens.

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Table 1 DDT and PCB in Antarctic snow from Doumer Island, January–February 1975

Depth (m)	Concentration (10^{-15} g g ⁻¹)			Σ DDT/PCB
	p,p'-DDT	p,p'-DDE	PCB	
0–0.5	500	130	300	2.1
0–0.5	550	58	170	3.6
0–0.5	440	54	30	16
0.5–1.0	450	67	57	9.1
1.0–1.5	1,300	62	220	6.2
1.3–1.6	2,700	220	1,200	2.4
3.5–4.0	4,000	270	400	11
4.0–4.5	3,000	240	680	4.8
4.5–5.0	2,800	170	430	6.9
5.0–5.5	3,400	380	810	4.7
5.5–6.0	2,100	210	280	13

the Antarctic Convergence^{2,3–8} and were detected in all eggs and tissues of seabirds examined from the subantarctic Auckland Islands, centred at 166°05'E, 50°40'S. These data have been used to support the hypothesis that the atmosphere is not the primary medium of transport of this class of pollutants to remote environments such as Antarctica¹. In the present paper we report the detection of PCB in the eggs of resident penguin species, by removal of unknown compounds previously interfering in the analysis, and in antarctic snow, by application of *in situ* extraction in the field of large volumes of melted snow. Equivalent levels and ratios of these pollutants in species resident north and south of the Convergence indicate atmospheric, rather than oceanic, transport to Antarctica.

In January 1975, a field camp was established on Doumer Island (63°35.5'W, 64°51.3'S) on the Antarctic Peninsula. Ninety-nine litre samples of melted snow were extracted *in situ* by passage through a glass column (length 30 cm, internal diameter 3 cm) packed with polyurethane foam plugs (81% polyether glycol, 19% toluene di-isocyanate, density 0.036 g cm⁻³, United Foam Company, Los Angeles). The packed columns and all equipment were cleaned in the laboratory with successive washes of acetone until background levels of DDT and PCB compounds were less than 10⁻¹⁴ g g⁻¹ in a 100-l sample; all equipment was wrapped tightly in clean aluminium foil until used in the field. Controls included columns transported to Antarctica, which were not opened, and which were returned to the laboratory for analysis. Samples obtained at depths greater than 2 m were obtained by excavating in the wall of a small crevasse. Results are presented in Table 1; analytical techniques have been reported elsewhere³. The concentrations of total DDT, ranging from 0.5 to 4 × 10⁻¹² g g⁻¹, are in the same range but somewhat higher than those previously reported by Peel¹ from an area considerably further south (26°42'W; 75°31'S). Concentrations of PCB, principally pentachlorobiphenyls, ranged between 0.03 to 1.2 × 10⁻¹² g g⁻¹; the median Σ DDT : PCB ratio was 6.2.

Elsewhere⁴, the hypothesis that the majority of the organochlorine pollutant residues in Antarctica have

Transfer of chlorinated biphenyls to Antarctica

PREVIOUS determinations of synthetic organic compounds in antarctic snow¹ and in tissues of species inhabiting the antarctic marine environment^{2–3} have shown the presence of DDT compounds in almost all samples examined, but the apparent absence of polychlorinated biphenyls (PCB). PCB, however, are ubiquitous marine pollutants north of

Table 2 PCB and DDE concentrations in eggs of penguins from the Antarctic Peninsula

Species	Date	Locality	N	% H ₂ O	% lipid	p,p'-DDE†	PCB†
<i>P. adeliae</i>	Jan 1970	Anvers Island	14	Not determined	11.8 (7.0–14.6)	137 (65–330)	81 (40–180)
	Dec 1973	Anvers Island	15	74.4 (69–80)	8.2 (5.5–10.6)	204 (86–310)	73 (27–160)
	13 Jan 1975	Anvers Island	12	73.0 (70–78)	9.7 (5.6–13.0)	139 (72–200)	56 (27–93)
<i>P. antarctica</i>	11 Jan 1975	Gibbs Island*	10	75.1 (69–78)	10.8 (4.9–16.4)	172 (90–380)	43 (21–80)
<i>P. papua</i>	15 Jan 1975	Doumer Island	8	75.0 (73–79)	9.0 (6.5–10.8)	168 (110–310)	72 (27–140)

*Approximately 55.6°W, 61.5°S.

†Lipid basis, 10⁻⁹ g g⁻¹. Geometric means with the range.

derived from local human activities has been examined and rejected; ratios among the compounds detected are not consistent with those predicted. Potential local sources of PCB contamination on Doumer Island included the small Chilean base Yelcho, 2 km distant from the sampling site, which has been occupied intermittently over the past few years, and Palmer Station, the much larger American base to the west on Anvers Island (64°3'W, 64°46.3'S). Local contamination of PCB on Anvers Island was shown by analysing four samples of surface snow obtained on February 13–16, 1975, 0.5–1.5 km from Palmer Station. This snow had a high content of particulates, principally soot, and contained from 4 to 10×10^{-12} g g⁻¹ of total PCB. We conclude that the PCB residues most probably derived from the frequent burning in the past of considerable quantities of waste materials at Palmer Station. A portion of the PCB residues detected on Doumer Island may therefore derive from local input.

Widespread distribution of PCB in the Antarctic was, however, demonstrated by the analysis of diverse members of the food web from separated localities. The results of the analysis of eggs of three species of penguins obtained between January, 1970, and January, 1975, are presented in Table 2. All extracts contained compounds, presumably of natural origin, which produced peaks on electron capture gas chromatograms which interfered with the detection of polychlorinated biphenyls. These compounds were destroyed, however, by rigorous saponification; a saponification side column was therefore used in the analysis of these samples⁵.

No significant differences in concentrations of *p,p'*-DDE or PCB were found among the three species obtained in 1975 (Wilcoxon 2-sample test, $P > 0.05$). PCB concentrations, however, were significantly lower ($P < 0.001$) in Adelie Penguin eggs obtained in 1975 than in those obtained in 1970. Median DDE:PCB ratio in the penguin eggs was 3.0: concentrations of *p,p'*-DDT in the eggs were usually a small fraction of the concentration of *p,p'*-DDE.

Other samples examined for the presence of PCB included the blubber of a leopard seal (*Hydrurga leptonyx*), which had been killed by killer whales (*Orcinus orca*) in the Gerlache Strait (62°10'W, 64°30'S) on October 30, 1975. Concentrations in the fat of *p,p'*-DDE, *p,p'*-DDT and PCB (principally pentachlorobiphenyls) were 48, 33 and 43×10^{-9} g g⁻¹, respectively. These levels are equivalent to those in the penguins, suggesting that this animal had not been feeding on penguins, but rather on a common food source such as krill. A sample of krill (*Euphausia* sp.), obtained at a depth of 250 m on October 31, 1975, at 60°27'W, 62°39'S with an Isaac Kidd midwater trawl, contained 14, 19 and 3×10^{-9} g g⁻¹ respectively of *p,p'*-DDE, *p,p'*-DDT and PCB (pentachlorobiphenyls) on an extractable lipid basis.

The concentrations of *p,p'*-DDE and PCB in penguin eggs from the Antarctic Peninsula are equivalent to levels recorded in four eggs of the rockhopper penguins (*Eudyptes crestatatus*) from the Auckland Islands in 1972–73. Moreover, DDE:PCB ratios in eggs of the Auckland island shag (*Phalacrocorax carunculatus*) ranged between 3.2 and 4.0; the ratio in the fat of a Hooker's sea lion (*Otaria hookeri*) was 5.1. Comparable DDE:PCB ratios were recorded in species from other areas of the New Zealand subantarctic and coastal New Zealand⁹. These ratios, typical also of the Antarctic, reflect the relative usage and environmental input of DDT and PCB compounds into the Southern Hemisphere¹⁰; in the Northern Hemisphere PCB compounds usually predominate over the DDT group in marine samples^{5–8}. Comparable levels of organochlorine contamination north and south of the Antarctic Convergence, which separates sharply defined and distinct water masses, indicate that the atmosphere is the dominant pathway of transport of both PCB and DDT compounds to the Antarctic.

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²⁴¹Americium in Mediterranean surface waters

LITTLE information is available to date on the occurrence of ²⁴¹americium in sea water, except for data obtained in some special areas such as in the vicinity of a nuclear reprocessing plant (Windscale, UK)¹ or in areas of close-in fallout from nuclear explosion tests (Bikini and Eniwetok Atolls, Pacific)². So far, only a few data of ²⁴¹Am obtained from one station in the North Atlantic have been published by Livingston *et al.*³. This is mainly due to analytical difficulties involved in determining extremely low concentrations of ²⁴¹Am in open ocean waters. However, since the inventory of ²⁴¹Am in radioactive wastes from nuclear fuel reprocessing is expected to exceed that of ²³⁹Pu plus ²⁴⁰Pu in the near future, it is important to start obtaining baseline data of ²⁴¹Am in oceanic waters now, in order to follow the future trend of concentration changes, if any, of this isotope.

We have determined the concentration of ²⁴¹Am in surface waters of the Mediterranean Sea, collected from several stations covered during the 1975 cruises. The detailed analytical procedures employed were described by Ballestra *et al.*⁴. Briefly, ²⁴¹Am together with plutonium isotopes were coprecipitated with mixed hydroxides and carbonates of calcium and magnesium, which are naturally present in seawater, from approximately 200 l of unfiltered seawater; a second coprecipitation was carried out by scavenging with ≈ 50 mg Fe³⁺. ²⁴¹Am was purified from plutonium isotopes as well as other interfering α -emitters such as U, Po, and Th, by cation and anion exchange and solvent extraction with HDEHP (di-2-ethylhexyl phosphoric acid-heptane); finally ²⁴¹Am was electrodeposited on to a stainless steel disk and α -spectrometrically determined by using a silicon surface barrier detector. The chemical yield of ²⁴¹Am for the separation procedure, determined by the initial addition of known amounts of ²⁴³Am, was 40–70%.

The results of these measurements are given in Table 1. In this table the results for ²³⁸Pu and ²³⁹+²⁴⁰Pu are also presented. Considering the errors involved in the measurements, the data in Table 1 show rather homogeneous distributions of these isotopes in the surface layer of the Mediterranean. On the basis of these data average concentrations of ²⁴¹Am, ²³⁸Pu and ²³⁹+²⁴⁰Pu in surface water of the Mediterranean are calculated

Table 1 Results of measurements of transuranic elements in Mediterranean surface waters

Position	Date of collection	Chlorinity (‰)	$^{239+240}\text{Pu}^*$ (fCi l $^{-1}$)	$^{238}\text{Pu}^*$ (fCi l $^{-1}$)	$^{241}\text{Am}^*$ (fCi l $^{-1}$)
43°39'N, 12°00'E	2 July '75	21.26	1.35 ± 0.06	0.06 ± 0.01	0.06 ± 0.01
43°11'N, 06°32'E	14 Sept '75	—	0.91 ± 0.07†	0.10 ± 0.03†	0.03 ± 0.01†
38°30'N, 06°30'E	17 Sept '75	20.56	0.77 ± 0.06	0.07 ± 0.02	0.08 ± 0.02
37°30'N, 06°30'E	18 Sept '75	20.78	0.93 ± 0.07	0.06 ± 0.01	0.09 ± 0.02
38°40'N, 12°00'E	20 Sept '75	20.12	1.04 ± 0.06	0.05 ± 0.01	0.05 ± 0.02
40°40'N, 11°40'E	20 Sept '75	21.18	1.09 ± 0.09	0.05 ± 0.01	0.07 ± 0.02
41°20'N, 11°30'E	21 Sept '75	21.11	0.95 ± 0.09	0.06 ± 0.02	0.05 ± 0.01
42°47'N, 09°25'E	21 Sept '75	21.13	1.1 ± 0.1	0.06 ± 0.02	0.03 ± 0.01
43°55'N, 09°00'E	22 Sept '75	21.17	1.10 ± 0.06	0.10 ± 0.02	0.05 ± 0.01

* Errors indicated are 1σ propagated errors.

†The water sample was filtered through HA Millipore (0.45 μm pore size).

to be 0.057 ± 0.007 , 0.068 ± 0.007 and 1.03 ± 0.05 fCi l $^{-1}$ respectively.

These average values give the following activity ratio

$$^{241}\text{Am}/^{239+240}\text{Pu} = 0.055 \pm 0.007$$

As to the $^{241}\text{Am}/^{239+240}\text{Pu}$ ratio only one value 0.033, obtained for the Lake Michigan water, has been reported⁸. The similar ratio for North Atlantic surface water calculated on the basis of the data presented by Livingston *et al.*³ is 0.2 ± 0.1 . Our ratio is in reasonable agreement with that given for the Lake Michigan water. It is believed to be representative at least for surface waters of the Mediterranean and possibly also for surface layers of oceans in the temperate zone of the Northern Hemisphere, since fallout delivery ratio as well as mechanisms of removal from the upper mixed layer of these isotopes are not likely to be substantially different among different oceanic areas in this zone.

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Phosphate adsorption on goethite (α-FeOOH)

SYNTHETIC goethite has been extensively studied as a model adsorbent in the search for a precise description of the chemical reactions involved in phosphate adsorption by soil minerals. Infrared studies^{1,2} have now firmly established that phosphate on goethite and other iron oxides results in a bridging binuclear complex of the type Fe—O—P—O—Fe. The infrared studies were, however, carried out on dried samples of phosphated goethite. We now report infrared studies on wet goethite films which

confirm that the binuclear complex exists in the presence of water.

Furthermore, infrared spectra of phosphate adsorbed on goethite show differences according to the suspension pH at which the sample was prepared^{1,3}. These probably arise from different states of protonation of the adsorbed phosphate, but the results have been difficult to interpret. The possibility of different states of protonation of adsorbed phosphate is suggested also by potentiometric titration measurements⁴⁻⁷ of the surface charges of phosphated and non-phosphated goethite and other minerals, but this method cannot distinguish between phosphate groups and other surface sites as locations for hydrogen ions. It seemed desirable to combine the two methods, using comparable samples, and by doing so we are now able to report an improved interpretation of the spectral changes caused by changes in the state of protonation of adsorbed phosphate on goethite.

The synthetic goethite was prepared by the usual methods⁸ (OH/Fe 1.5, 50-h ageing). The phosphate adsorption capacity of 197 μmol g $^{-1}$ at pH 3.3 indicated⁹ a surface area of ~ 80 m² g $^{-1}$. This corresponds to ~ 400 μmol singly co-ordinated OH $^{-}$ or H₂O (A—type²)/g of goethite.

Phosphated goethite suspensions were prepared for infrared spectroscopy using almost completely adsorbed amounts of phosphate (see Table 1). The samples were drained to water contents of ~ 1.0 g of H₂O/g of goethite, on AgCl plates. The thickness of the water film around each goethite crystal was estimated to be 10–15 nm at this water content.

The infrared spectra of the goethite suspensions phosphated with H₃PO₄ (pH 3.6 or 5.1) showed two phosphate bands near 1,100 and 1,000 cm $^{-1}$ (Table 1). These bands arise from the P=O and P—O—(Fe) stretching vibrations of phosphate adsorbed in a binuclear bridging complex (FeO)₂POOH (refs 2, 3). Drying caused the P=O band to shift to higher frequencies as the influence of hydrogen bonded water dwindled. On treating the evacuated complexes with D₂O and re-evacuating, the phosphate bands shifted to different frequencies, indicating that the phosphate group was protonated.

When NaH₂PO₄ (pH 8.1) or Na₂HPO₄ (pH 9.7) was added to the goethite suspensions, phosphate bands were observed near 1,080 and 1,040 cm $^{-1}$. Drying caused the 1,080 cm $^{-1}$ band to shift to a higher frequency (air dry, 1,105 cm $^{-1}$; evacuated, 1,192 cm $^{-1}$) while the 1,040-cm $^{-1}$ band shifted to a lower frequency (1,000 cm $^{-1}$) characteristic of the binuclear complex. The 1,080 and 1,040 cm $^{-1}$ bands returned immediately on rewetting the goethite films. Since it is unlikely that rearrangement to a monodentate species could occur so readily, the most

Table 1 Absorption bands (ν(cm $^{-1}$)) of phosphated goethite

Phosphate added (μmol g $^{-1}$)	Suspension pH	In suspension			Evacuated		D ₂ O, evacuated	
		P = O	PO ₂ $^{--}$	P—O—(Fe)	P = O	P—O—(Fe)	P = O	P—O—(Fe)
200 H ₃ PO ₄	3.6	1,115	—	1,000	1,192	996	1,185	1,013, 991
100 H ₃ PO ₄	5.1	1,100	—	1,000	1,192	1,000, 986	1,183	1,000
100 NaH ₂ PO ₄	8.1	—	1,086	1,042	1,192	1,000	1,183	1,000
50 Na ₂ HPO ₄	9.7	—	1,070	1,040	1,192	1,000, 986	1,183	1,000

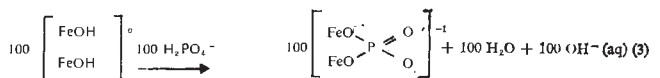
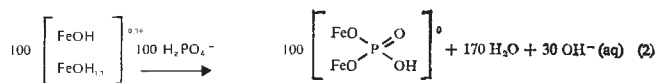
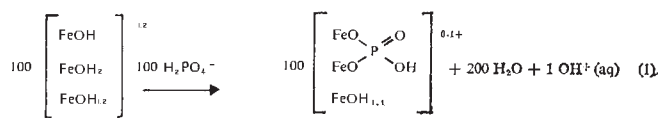
plausible explanation for the 1,040- and 1,080-cm⁻¹ bands is that the phosphate is in an ionised binuclear form (FeO)₂POO⁻Na⁺.

The phosphate bands for evacuated goethite from high pH suspensions are similar to the phosphate bands for evacuated goethite from low pH suspensions (Table 1), and the bands show similar shifts after exchange with D₂O. These results show that the (FeO)₂POONa complex becomes protonated when evacuated, probably by Na⁺ exchanging with H⁺ from Lewis acid sites on the (010) faces².

The potentiometric titration curves of goethite suspensions in NaCl solutions, with and without phosphate, are shown in Fig. 1. The phosphate addition was 100 µmol NaH₂PO₄ per g goethite, which was virtually completely adsorbed, and thus the results do not require corrections for the titration of non-adsorbed phosphate.

Surface-charge data can be obtained by choosing an origin which is the zero point of charge, located by the common point of intersection of the titration curves⁴, at pH 8.1 for non-phosphated goethite, and at pH 5.1 for phosphated goethite. A similar shift in the zero point of charge was observed for haematite, changing from pH 8.9 (non-phosphated) to pH 5.3 (phosphated)¹⁰. Other anions have similar effects⁴.

Surface-charge data obtained from the potentiometric titration curves in 0.01 M NaCl were used to construct equations (1), (2) and (3) which refer to pH 3.6, 5.1 and 8.1 respectively (selected to correspond with the spectroscopic sample preparation conditions). The equations are written in numbers of µmol, to facilitate comparison with experimental measurements.



The OH₂, OH, OH_{1.2} and so on, represent singly coordinated species² which can have an average state of protonation ranging between 1.0 and 2.0 depending on pH and ionic strength. Other species, which are bridging in the coordination shells of the iron atoms on the goethite surfaces, are not shown. The coordination (bridging binuclear) and the state of protonation of the adsorbed phosphate shown agree with the results from infrared spectroscopy.

The equations (1)–(3) predict the release of 10 µmol H⁺ per g goethite, 30 µmol OH⁻ per g goethite, and 100 µmol OH⁻ per g goethite at the selected pH values, 3.6, 5.1 and 8.1 respectively. To test these predictions goethite suspensions and phosphate (100 µmol g⁻¹) solutions at these pH values were flushed with nitrogen, mixed, and allowed to equilibrate under a nitrogen atmosphere. They were then titrated to the original pH values and the results showed that 16 µmol H⁺, 23 µmol OH⁻, and 80 µmol OH⁻ respectively were released during the adsorption reaction, in fair agreement with the predicted values. The consistency between potentiometric titration data and the findings from infrared spectroscopy support our conclusion that adsorbed phosphate on goethite is mostly protonated (HPO₄²⁻) at pH 3.6, and mostly deprotonated (PO₄³⁻) at pH 8.1.

Similar results were obtained from titrations carried out without nitrogen flushing, but the interpretation is more difficult because carbonate ions are adsorbed on goethite in

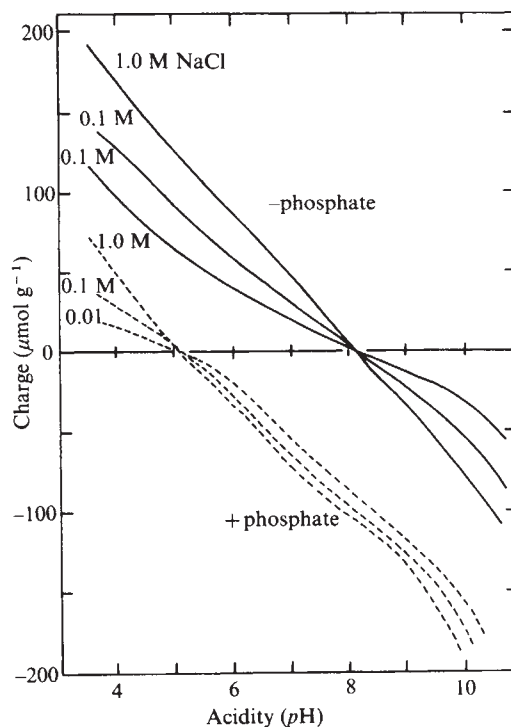


Fig. 1 Surface charges of goethite (—) and phosphated goethite (---), from potentiometric titrations of suspensions in various NaCl concentrations. Phosphated goethite was prepared by adding 100 µmol NaH₂PO₄ per g goethite.

these conditions¹¹. The apparent zero points of charge of goethite and phosphated goethite were pH 8.9 and 5.1 respectively, when nitrogen flushing was not used.

The equations (1)–(3) do not represent different types of adsorption reaction or different types of sites. In accordance with a proposed general model for anion adsorption on oxide surfaces¹², equations (1)–(3) represent average states of protonation of surface ligands. For example, at some pH value between 5 and 8 we may expect to find equal numbers of protonated and deprotonated adsorbed phosphate.

These results extend the work reported by others^{5–7} in two important respects. First, we have used infrared spectroscopy to examine the coordination and state of protonation of adsorbed phosphate, in wet pastes and *in vacuo*. Second, we have found that equations based on surface-charge measurements by potentiometric titration, and on infrared spectroscopy results, give reasonable agreement between predicted and observed H⁺ release or OH⁻ release during phosphate adsorption on goethite.

Recent work suggests that binuclear phosphate complexes occur also on gibbsite surfaces^{13,14}.

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Possible interstadial and interglacial pollen floras from Teindland, Scotland

THIS paper reports new investigations carried out on the Teindland soil profile first described by E. A. FitzPatrick¹. The section (grid ref. NJ 3297 8570) lies at an altitude of about 101 m OD in a disused quarry within Teindland Forest, 5 km south-west of Fochabers in Grampian Region, Scotland. The present study presents evidence which suggests that a fossil microflora contained within the soil can be assigned to two distinct phases of geological time, the Middle Devensian interstadial and the Ipswichian stage. This is augmented by stratigraphic analyses which suggest that the site is of wider significance for the Quaternary chronology of the area than has been appreciated.

The stratigraphy of the Teindland profile as described by FitzPatrick may be summarised as follows. The section showed at the top a semi-podsol in 2–2.4 m of sandy till and outwash gravel which overlay a fossil iron podsol developed in fluvio-glacial outwash. The upper part of the buried soil which was originally the organic and leached layers, had been subjected to solifluction processes, producing thin bands of black and grey material. A radiocarbon assay from the organic black bands rendered a date of 28,140±480 and –450 years b.p. (NPL-78). FitzPatrick interpreted the history of the section thus: (1) Glaciation (Riss? = Wolstonian?) followed by the deposition of fluvio-glacial deposits. (2) Pedogenesis in the fluvio-glacial deposits of (1) during an interglacial (= Ipswichian?) to produce a well developed iron podsol. (3) Periglacial conditions causing disturbance and solifluction of the surface layer. (4) A second period of glaciation (Würm? = Devensian?) during which the soil was buried after 28,140 b.p. beneath till and outwash. (5) Holocene (= Flandrian) pedogenesis in the upper part of the Devensian deposits.

Pollen analysis and limited stratigraphical investigation were carried out on the Teindland profile in order to test FitzPatrick's interpretation of the site's evolution, and to establish whether evidence could be found for environmental conditions in the largely unknown Middle Devensian and Ipswichian stages in Scotland.

The stratigraphy at the point of sampling is shown in Fig. 1. Samples for pollen analysis were treated by Erdtman's acetolysis and hydrofluoric acid digestion². Preparations were mounted in silicone oil. There was a general scarcity of pollen and the lowest count for which there is percentage representation in the diagram is 126 land pollen grains (320 cm). Three levels contained no pollen (230, 289 and 310 cm) and elsewhere only traces were found. To facilitate discussion, the pollen diagram (Fig. 2) is zoned biostratigraphically (Table 1)³, though it will be apparent that the presence of no more than traces of pollen at some levels makes boundary location somewhat arbitrary, and the zones are partly discussed out of strict numerical sequence.

As might be expected from a profile which has had a long and varied history, the contained pollen record is not simple. The surface sample (zone T-6) with its *Pinus*, *Calluna vulgaris* and Gramineae assemblage differs from the spectra in underlying zone T-5 which is presumed to

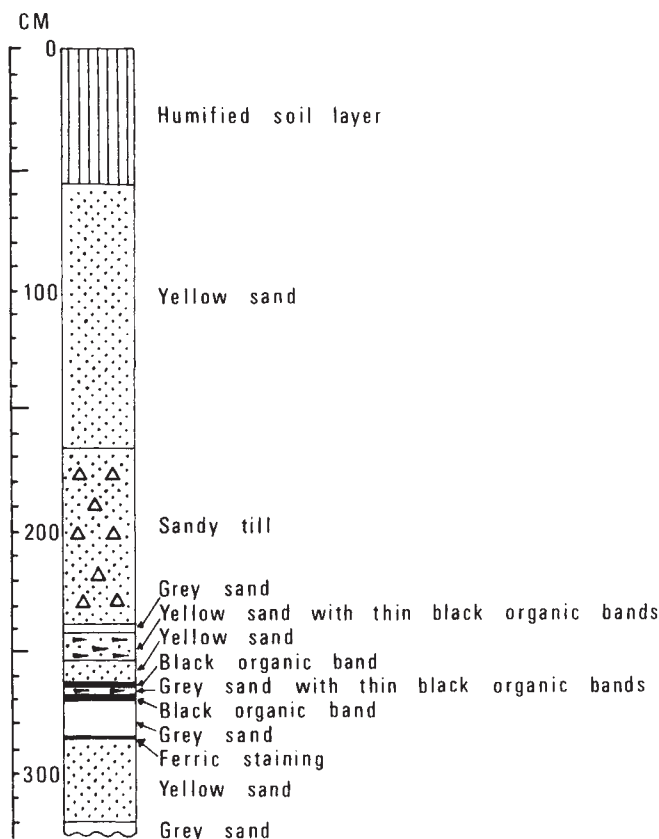


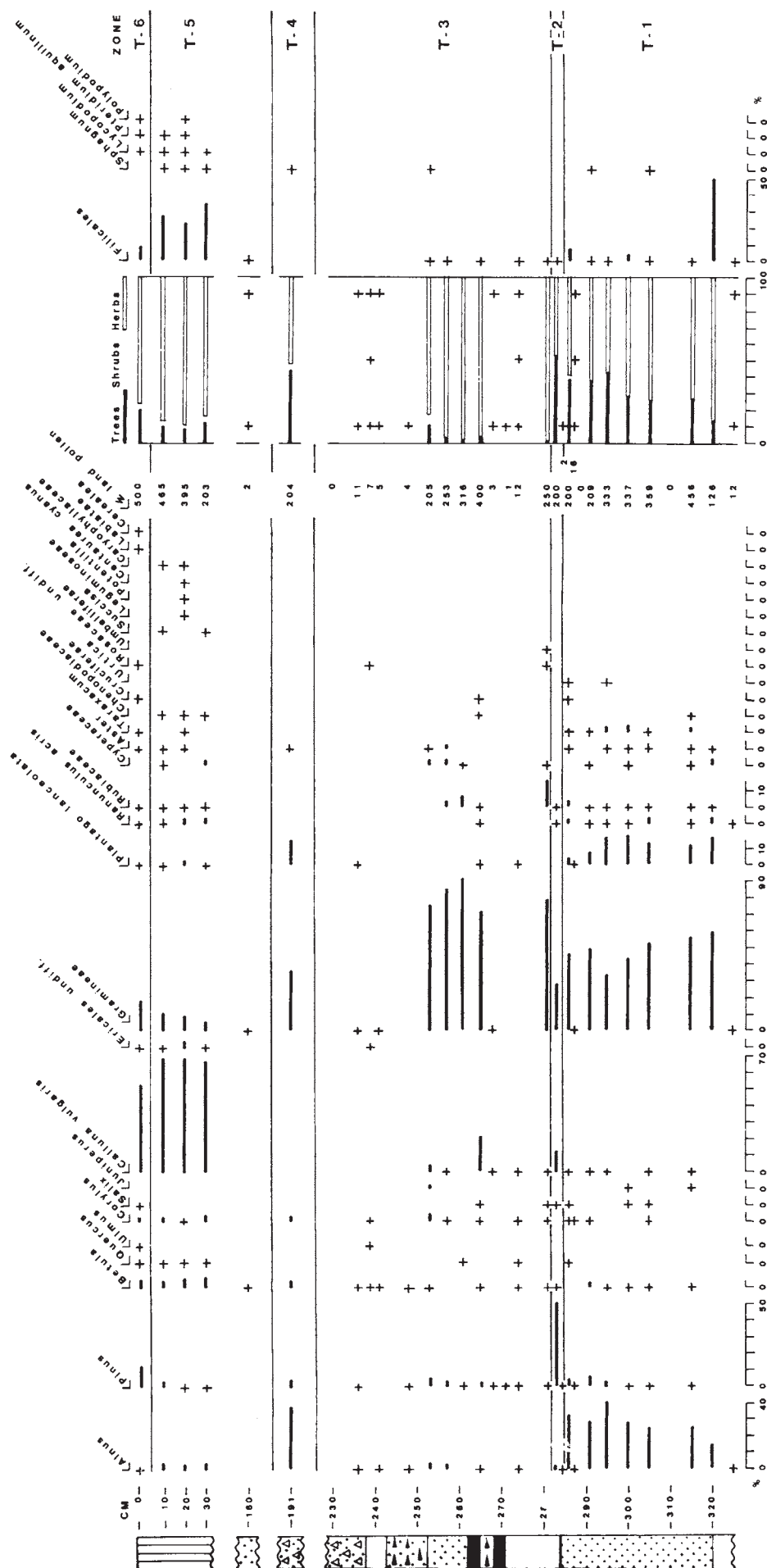
Fig. 1 Stratigraphy.

represent the heath-type vegetation cover prior to the planting of *Pinus sylvestris* woodland about forty years ago. The homogeneous nature of the spectra in zone T-5 is probably due to a process of microfaunal mixing of pollen and/or downflow of pollen through the soil profile^{4,5}. Zones T-5 and T-6 are also quite different from zone T-4, represented by a spectrum obtained from sand within the till (the only till sand sample to produce a large pollen count). This suggests that any downflow of microfossils through the upper soil over the last forty years, or through the till at any stage, has been negligible.

The *Alnus*, Gramineae, *Plantago lanceolata* pollen assemblage of zone T-4 is remarkably similar to the spectra of basal zone T-1. This is a feature of the utmost interest. These zones alone contain high frequencies of *Alnus* and *Plantago lanceolata* pollen. The location of zone T-1 below organic levels dated to the Middle Devensian (zone T-3), and the arboreal content of zone T-1 lead us to infer that these spectra represent a record of part of an interglacial landscape within north-east Scotland, chronologically this would suggest the Ipswichian stage. The location of zone T-4 above the radiocarbon-dated horizon may be explained by the fact that the passage of glacial ice over the area after 28,140 b.p. led to the incorporation within the till of interglacial deposits similar in age to those still preserved at Teindland. It is not possible to say how near the postulated palaeosol was to the site under investigation. The differences in representation between the similar spectra

Table 1 Pollen assemblage zones

T-6	<i>Pinus</i> – <i>Calluna</i> –Gramineae zone
T-5	<i>Calluna</i> zone
T-4	<i>Alnus</i> –Gramineae– <i>Plantago lanceolata</i> zone
T-3	Gramineae zone
T-2	<i>Pinus</i> – <i>Calluna</i> –Gramineae zone
T-1	<i>Alnus</i> –Gramineae– <i>Plantago lanceolata</i> zone



would result from the spatial disparity between the polleniferous deposits within the interglacial landscape, and possibly differential preservation.

The vegetation at this stage of the posited interglacial would appear to have been light woodland dominated by *Alnus*, the latter especially in the wetter areas. Pine may have been present though the low frequencies of less than 6% might suggest long distance wind transport. This agency or differential pollen destruction⁶ might also be evoked for the traces of *Betula*, *Quercus*, and *Corylus pollen*. The open areas of the landscape would seem to have been dominated by Gramineae, *Plantago lanceolata*, and ferns. The assemblage is somewhat unusual, and this may be in part a function of pollen preservation, though the dominants in zone T-1 are known from proposed Ipswichian deposits in East Anglia^{7,8}. Conformity with the English pattern would not necessarily be expected but the spectra in zone T-1, especially with regard to the *Alnus* and *Plantago lanceolata* frequencies, bare a striking resemblance to the pollen content of the zone Ip III deposits at Swanton Morley in Norfolk, which have been assigned to an "Ipswichian late-temperate zone" immediately preceding an "Early Devensian zone"⁹. The homogeneous spectra of zone T-1 are possibly due to downwash of grains from an interglacial soil surface which may have been at any level below or within the supposed soliflucted horizons of zone T-3.

The lone spectrum of zone T-2 might be interpreted as depicting a later phase of the interglacial. It quite possibly reflects the vegetational conditions pertaining at the time of podsolisation and might be compared to a certain extent with the present-day plant cover of *Pinus sylvestris*, *Calluna vulgaris* and Gramineae spp. overlying the semi-podsol developed above the till. This change between zones T-1 and T-2 may indicate a retrogressive vegetation succession¹⁰ as has been noted in palynological investigations of podsol profiles elsewhere¹¹. The sharp transition between zones T-1 and T-2 (and, indeed T-3) might appear enigmatic. The boundary between T-1 and T-2 occurs at a stratigraphic break where the thin ferric horizon separates yellow and grey sand. Whether this has any implications in terms of deposit age or pollen accumulation or is merely a coincidental feature arising from the podsolisation process is unclear. Signs of microfossil movement within this section of the profile are not apparent; the spectra are quite distinct with no obvious signs of homogenisation. The question of pollen fixation with abrupt changes between horizons, as opposed to translocation, downwash and homogenisation of pollen in leached mineral profiles, is a subject of long and continuing debate^{5,12}. The Teindland site thus far discussed would appear to provide evidence for the operation of both processes at different times in the development of the pollen profile.

The sample at the base of zone T-3 (281 cm) with its 97.6% herbaceous pollen consisting mainly of Gramineae and Rubiaceae spp., may indicate the cold climatic conditions at the end of the proposed interglacial stage. Alternatively, it possibly belongs chronologically to the polleniferous section in the middle zone T-3 among the soliflucted organic layers. Here is also to be found a predominance of open land taxa. Gramineae pollen dominates throughout with high frequencies of *Calluna vulgaris* pollen at 265 cm and some *Alnus*, *Corylus* and *Juniperus* pollen at 253 cm. It is possible that the representation of these types is due to a warming of local climatic conditions, though some of the pollen may be derived from the pollen within the till by downwash through the unpodsolised sand above the organic layers. Another possible mechanism could be wind transport, an agency which might be particularly evoked for the low frequencies of *Pinus* pollen. The traces of pollen found within and on either side of these probable interstadial spectra may represent some derived

types or be the result of poor pollen preservation. The microflora of zone T-3 indicate cold climatic conditions. This would be consistent with the Middle Devensian radiocarbon date of 28,140 BP, and might be compared with the more oceanic interstadial conditions inferred from the site at Tolsta Head, Island of Lewis, where a polleniferous limnic deposit yielded a radiocarbon date of 27,333±240 BP (SSR-87)¹³.

The interpretation of the palynologically-derived data presented above, corroborates FitzPatrick's stratigraphic suppositions¹ and would seem to provide evidence for the existence of, and conditions within the Middle Devensian and Ipswichian stages in north-east Scotland. Only two polleniferous deposits of pre-Late Devensian age have been reported from Scotland^{13,14}. The importance of the Teindland site in providing possible evidence for the first Scottish Ipswichian stage flora and only the second Middle Devensian microfossil assemblage will be obvious.

In addition, detailed stratigraphic analysis of the till using fabric, particle size and shape criteria has been carried out, demonstrating a glacial origin and probable correlation with the last full glacial conditions in this area. A preferred orientation of pebbles of 110°–120°, accords with the accepted direction of ice flow derived independently from a study of ice directed landforms and melt-water channels¹⁵. This finding tends to contradict a report that the till horizon at Teindland is a soliflucted deposit¹⁶ and adds further weight to FitzPatrick's original interpretation¹, since the deposit has none of the sedimentological characteristics usually associated with soliflucted till, and orientation measurements do not indicate slope controlled deposition. The particle size and shape statistics are similar to results obtained from other sections of the same till in north-east Scotland (Chester unpublished). It has been argued that there is no evidence for more than one phase of glaciation in north-east Scotland and that all till deposits are of the same age^{15,17,18}. If this reasoning is accepted, then the Teindland site provides a maximum age for the onset of full glacial conditions in this area.

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Geography and dispersal of Galapagos Islands vascular plants

NEARLY every naturalist who has dealt with the Galapagos Islands since Darwin¹ has pointed out the flora's obvious geographical affinities with tropical America, stressing relationships with South America, Mexico, Central America and the West Indies. But as the tropical American flora has become better known, most Galapagos species once thought to be only Mexican, Central American or West Indian in their extra-Galapagean distributions also have been found to occur in northern and western tropical South America (for example, the widespread *Zanthoxylum fagara* (L.) Sarg., Rutaceae) or to have been incorrectly identified (for example, the endemic *Chamaesyce viminea* (Hook. f.) Burch, Euphorbiaceae). Svenson² first indicated the flora's lack of affinity with Mexico and Central America. The study reported here is the first to present evidence that West Indian relationships do not exist. After publication of the *Flora of the Galapagos Islands*³ and several subsequent papers⁴⁻¹², it is now also possible to quantify the geographical relationships of the vascular flora and to study the plants' dispersal mechanisms. This study shows the

endemics have their closest known relatives in South America as well. The geographical evidence is overwhelming that the indigenous Galapagos flora has been derived almost totally from South America, most probably from the Andean region. With only eight exceptions, all non-endemic species or the presumed progenitors of the endemics occur in this region.

How did the plants get there? As a result of this study, I estimate that a minimum of 378 original introductions of plant disseminules can account for the 522 indigenous vascular plants of the archipelago (Table 2).

It is no surprise that these oceanic islands have received the bulk of their flora by means of long-distance dispersal through the agency of birds, with fruits, seeds or vegetative disseminules either attached externally or carried internally. The importance of long-distance bird dispersal of disseminules to oceanic islands is well documented^{13,14}. I estimate that 144 original introductions (64%) arrived internally (for example, most Cyperaceae), 34 (15%) in mud attached to birds (for example, *Azolla microphylla* Kaulf., Azollaceae), 28 (12%) attached by viscid structures of seeds or fruits (for example, *Elipta alba* (L.) Hassk., Asteraceae) and 19 (8%) attached mechanically (for example, *Bidens riparia* HBK., Asteraceae).

Hooker¹⁵, Robinson¹⁶, and Stewart^{17,18} indicated the prob-

Table 1 Geographical relationships of the indigenous vascular plants of the Galapagos Islands

	Endemic	Neotropical	Pantropical	Andean	Mexico and Central America	South America	Total
Pteridophytes	8	52	14	15		2	91
Monocotyledons	20	38	22	3			83
Dicotyledons	208	65	26	43	4	2	348
Total	236 (45%)	155 (30%)	62 (12%)	61 (12%)	4 (1%)	4 (1%)	522

Geographical areas are defined as follows: endemic (occurring only in the Galapagos Islands); neotropical (distributed generally in the American tropics); pantropical (distributed in both the Old and New World tropics); Andean (occurring only in western South America from Venezuela to Chile, generally or in part); Mexico and Central America (occurring only in Mexico and/or Central America); South America (occurring only in extra-tropical South America).

flora's relationships to be with adjacent South America and and that birds have had the most important role in plant dispersal to the islands.

The known extra-Galapagean distributions for the archipelago's indigenous vascular plants (that is, those not introduced by man's activities) are given in Table 1, which includes the absolute numbers and percentages of the vascular flora's distribution by geographical region. The numbers of taxa include all species, subspecies and varieties, but forms are excluded. A complete list of taxa and discussion of each will be published elsewhere. Further refinements in the knowledge of extra-Galapagean distributions may alter these figures slightly.

Ninety-nine per cent of the non-endemic vascular plant taxa in the Galapagos Islands also occur in South America. The remaining 1% occurs in Mexico and Central America. All but four of 286 indigenous non-endemic taxa in the islands are known from the land area nearest the archipelago, which at its closest is about 800 km away. The

ability that much of the flora was derived in this way, although they presented little data to support this viewpoint. Seabirds have been hypothesised as being instrumental in the early colonisation of the archipelago by other organisms¹⁹. Both seabirds and shorebirds have played an important role in plant dispersal, with migrants and casual visitors perhaps the most important. "More species have been recorded as migrants or vagrants than breed within the islands, and during the northern winter migrant birds form a very important part of the Galapagos avifauna"²⁰. Many of these are seabirds and shorebirds. The percentage of those disseminules having arrived on or in birds is greater (to 77%) when flowering plants only are considered.

In contrast to the figures of Table 2 and those given above are those of Carlquist^{13,14}, which were derived from Stewart's flora¹⁷ and based on an estimate of 308 original introductions. Carlquist determined that the flora had been derived as follows: 73% by birds (27.7% internal, 22.8% mechanical attachment, 13.7% in mud, 8.5% viscid attach-

Table 2 Original introductions that have resulted in the present vascular plant flora of the Galapagos Islands

Introduced	Birds	Man	Wind	Oceanic drift	Total
Pteridophytes	1		86		87
Monocotyledons	58	38	14	2	112
Dicotyledons	166	143	18	33	360
Total	225 (40%)	181 (32%)	118 (21%)	35 (6%)	559
Total for natural introductions	225 (60%)		118 (31%)	35 (9%)	378

ment); 23% by drift (21.8% frequent drift, 1.3% rare drift or rafting), and 4% by wind.

His figures and mine are close for overall dispersal by birds, but they are reversed for wind dispersal and drift. Although floating vegetation originating on the mainland has been reported from the vicinity of the archipelago²¹⁻²³, or even cast up on its beaches^{18,24}, drift has had a minor role in plant colonisation of the Galapagos. Those species that have arrived in this way are all members of the mangrove, beach or salt flat associations. I can find no evidence for any vascular plant species having arrived by rafting.

The small number of species that have been derived through oceanic drift is not surprising. Although drift-dispersed species have relatively high immigration rates in comparison with those which are dispersed by birds and wind, very few species are adapted to this mode of introduction. In addition, their higher immigration rates and the small numbers of habitats available to them in the Galapagos also reduce the opportunities for endemism in this group²⁵. The role of man in the introduction of exotic species into the islands is surprising (Table 1). Even Hooker¹⁵ remarked on the number of alien species that man had added to the flora by the time of Darwin's visit in 1835. Man has introduced 124 weeds by the latest count, and 57 of his cultivated exotics have escaped and now reproduce themselves in the wild. Today *Homo sapiens* has replaced birds as the most important factor in the dissemination of plants to the Galapagos. The deleterious effects on the native flora of some of the species introduced by man are just beginning to be appreciated^{6,19,26-28}.

Recent potassium-argon studies²⁹ indicate that the archipelago has a probable maximum age of 3 Myr. This means that the arrival and establishment of one successful disseminule about every 7,900 yr would account for the present indigenous flora. Introduction and subsequent extinction cannot be estimated, but they certainly have occurred as well. This is a higher rate of introduction than Fosberg's³⁰ estimate of one successful disseminule every 20-30,000 yr to account for the derivation of the indigenous vascular flora of the Hawaiian Islands, but the latter are much further from their source areas than are the Galapagos.

Such a relatively high rate of introduction is not surprising. These volcanic islands are a jumble of open pioneer habitats inhibited by a weedy flora. As others have pointed out, dispersal is only half the battle; establishment is the other half. In the Galapagos, those plants that have become established are almost all weedy, a phenomenon first recognised by Darwin¹. The chance of immigrants surviving in any specific locality is greater initially than later when more closed communities have evolved. Weedy plants, being adapted to open habitats, have been at an advantage when their disseminules reached the Galapagos Islands. Their offspring have given us the present flora.

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Hearing lips and seeing voices

MOST verbal communication occurs in contexts where the listener can see the speaker as well as hear him. However, speech perception is normally regarded as a purely auditory process. The study reported here demonstrates a previously unrecognised influence of vision upon speech perception. It stems from an observation that, on being shown a film of a young woman's talking head, in which repeated utterances of the syllable [ba] had been dubbed on to lip movements for [ga], normal adults reported hearing [da]. With the reverse dubbing process, a majority reported hearing [bagba] or [gaba]. When these subjects listened to the soundtrack from the film, without visual input, or when they watched untreated film, they reported the syllables accurately as repetitions of [ba] or [ga]. Subsequent replications confirm the reliability of these findings; they have important implications for the understanding of speech perception.

To further confirm and generalise the original observation, new materials were prepared. A woman was filmed while she fixated a television camera lens and repeated ba-ba, ga-ga, pa-pa or ka-ka. Each utterance was repeated once per second, with an interval of approximately 0.5 s between repetitions. From this master recording four dubbed video-records were prepared in which the original vocalisations and lip movements were combined as follows: (1) ba-voice/ga-lips; (2) ga-voice/ba-lips; (3) pa-voice/ka-lips; (4) ka-voice/pa-lips. Dubbing was carried out so as to ensure, within the temporal constraints of telerecording equipment, that there was auditory-visual coincidence of the release of the consonant in the first syllable of each utterance. Each recording comprised three replications of its auditory-visual composite. Four different counterbalanced sequences of recordings (1)-(4) were prepared, each with a ten-second gap of blank film between successive segments. The recordings were suitable for relay via a 19-inch television monitor; audio-visual reproduction was of good quality.

Twenty-one pre-school children (3-4 yr), 28 primary school children (7-8 yr) and 54 adults (18-40 yr) were tested. The adult sample was predominantly male; there were approximately equal numbers of boys and girls in the younger samples. Subjects were individually tested under two conditions: (1) auditory-visual, where they were instructed to watch the film and repeat what they heard the model saying, and (2) auditory only, where they faced away from the screen and again had to repeat the model's utterances. Every subject responded to all four recordings ((1)-(4) above) under each condition, each time in a different sequence; sequence of presentation was counterbalanced across subjects.

For the purpose of analysis, a correct response was defined as an accurate repetition of the auditory component of each recording. Under the auditory-only condition accuracy was high, with averages of 91, 97 and 99% for pre-school, school age and adult subjects respectively; errors were unsystematic. Under the auditory-visual condition, where subjects heard the original soundtrack, errors were substantial. For pre-school subjects

Table 1 Stimulus conditions and definition of response categories from auditory-visual condition

Stimuli		Response Categories				
Auditory component	Visual component	Auditory	Visual	Fused	Combination	Other
ba-ba	ga-ga	ba-ba	ga-ga	da-da	—	—
ga-ga	ba-ba	ga-ga	ba-ba	da-da	gabga bagba baga gaba	dabda gagla <i>etc.</i>
pa-pa	ka-ka	pa-pa	ka-ka	ta-ta	—	tapa pta kafta <i>etc.</i>
ka-ka	pa-pa	ka-ka	pa-pa		kapka pakpa paka kapa	kat kafa kakpat <i>etc.</i>

average error rate was 59%, for school children 52% and for adults 92%.

Subsequent analysis was confined to detailed consideration of responses to the auditory-visual presentations. Responses were first categorised according to the operational definitions illustrated in Table 1.

The meaning of 'auditory' and 'visual' categories is self-evident. A 'fused' response is one where information from the two modalities is transformed into something new with an element not presented in either modality, whereas a 'combination' response represents a composite comprising relatively unmodified elements from each modality. Responses which could not be unambiguously assigned to one of these four categories were allocated to a small, heterogeneous 'other' category. Table 2 presents the percentage of responses in each category.

Table 2 shows that the original observation of the effect of [ba]/[ga] presentations on adult responses is highly replicable; 98% of adult subjects gave fused responses to the ba-voice/ga-lips presentation and 59% gave combination responses to its complement. The effect is also generalisable, at least to other stop consonants; 81% of adults gave a fused response to pa-lips/ka-voice and 44% gave combination responses to its complement. The effects, however, are more pronounced with [ba]/[ga] than with [pa]/[ka] combinations; the latter comment applies to all ages.

The data in Table 2 also illustrate that the auditory perception of adult subjects is more influenced by visual input than is that of subjects in the two younger groups; the latter do not differ consistently from each other. It is interesting to note that where responses are dominated by a single modality, this tends to be the auditory for children and the visual for adults. However, it should also be noted that the frequency of fused responses to ba-voice/ga-lips, and pa-voice/ka-lips presentations is at a substantial level for

pre-school and school children alike. These auditory-visual illusions, therefore, are observable across a wide age span, although there clearly are age-related changes in susceptibility to them, particularly between middle childhood and adulthood.

Appropriate analyses confirm that the various effects reported for the auditory-visual condition are statistically significant. Alone, however, the data fail to testify to the powerful nature of the illusions. We ourselves have experienced these effects on many hundreds of trials; they do not habituate over time, despite objective knowledge of the illusion involved. By merely closing the eyes, a previously heard [da] becomes [ba] only to revert to [da] when the eyes are open again.

Contemporary, auditory-based theories of speech perception are inadequate to accommodate these new observations; a role for vision (that is, perceived lip movements) in the perception of speech by normally hearing people is clearly illustrated. Our own observations and those of others¹ indicate that, in the absence of auditory input, lip movements for [ga] are frequently misread as [da], while those for [ka] are sometimes misread as [ta]; [pa] and [ba] are often confused with each other but are never misread as [ga, da, ka or ta]. It is also known that, in auditory terms, vowels carry information for the consonants which immediately precede them². If we speculate that the acoustic waveform for [ba] contains features in common with that for [da] but not with [ga], then a tentative explanation for one set of the above illusions is suggested. Thus, in a ba-voice/ga-lips presentation, there is visual information for [ga] and [da] and auditory information with features common to [da] and [ba]. By responding to the common information in both modalities, a subject would arrive at the unifying percept [da]. Similar reasoning would account for the [ta] response under pa-voice/ka-lips presentations.

By the same token, it could be argued that with ga-voice/ba-lips

Table 2 Percentage of responses in each category in the auditory visual condition

Stimuli		Subjects	Responses				
Auditory	Visual		Auditory	Visual	Fused	Combination	Other
ba-ba	ga-ga	3-5 yr (n=21)	19	0	81	0	0
		7-8 yr (n=28)	36	0	64	0	0
		18-40 yr (n=54)	2	0	98	0	0
ga-ga	ba-ba	3-5 yr (n=21)	57	10	0	19	14
		7-8 yr (n=28)	36	21	11	32	0
		18-40 yr (n=54)	11	31	0	54	4
pa-pa	ka-ka	3-5 yr (n=21)	24	0	52	0	24
		7-8 yr (n=28)	50	0	50	0	0
		18-40 yr (n=54)	6	7	81	0	6
ka-ka	pa-pa	3-5 yr (n=21)	62	9	0	5	24
		7-8 yr (n=28)	68	0	0	32	0
		18-40 yr (n=54)	13	37	0	44	6

and ka-voice/pa-lips combinations the modalities are in conflict, having no shared features. In the absence of domination of one modality by the other, the listener has no way of deciding between the two sources of information and therefore oscillates between them, variously hearing [babga], [papka], and so on.

The *post facto* nature of this interpretation is acknowledged. More refined experimentation is required to clarify the nature and ontogenetic development of the illusions and their generality needs further investigation. We are at present working on these issues but believe that the finding now reported are of some interest in their own right.

Full details of the dubbing procedure are available from us. We thank the staff of the University of Surrey AVA Unit for their technical assistance in preparing materials and also Susan Ballantyne whose lip movements we filmed.

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Squeezing speech into the deaf ear

DEAFNESS caused by attenuation at the middle ear—conduction deafness—can be ameliorated by hearing aids and surgical procedures, but damage to the cochlea presents more severe problems¹. We are altering the amplitudes of different parts of the speech wave relative to one another, so that the features normally determining intelligibility are selectively amplified. Tests using speech modified in this way will show whether major aspects of speech processing are unchanged by sensorineural deafness, and, if so, whether the improvement is large enough to justify the use of such processing in hearing aids.

The principal characteristics of sensorineural deafness due to cochlear damage are: (1) loss of sensitivity to high frequencies; (2) tinnitus—whistling or hissing sounds; (3) recruitment—reduction of dynamic range for intensity, the threshold for detection being raised while the maximum acceptable intensity is near normal. Both conduction and sensorineural deafness can be simulated in the normal ear^{2,3}; conduction deafness by ear plugs and sensorineural deafness by adding masking white noise (that is, noise containing equal power in all audible frequencies). Linear amplification will compensate for the signal attenuation of conduction deafness. It cannot, however, overcome loss of hearing associated with the limited dynamic range of the damaged cochlea. Standard hearing aids applied in the latter case can amplify the peak sound intensities above acceptable limits, overloading the middle ear to produce distortion, discomfort and sometimes pain without giving adequate intelligibility to normal speech. They may also produce further damage to the hair cells^{4,5}.

Fortunately, the energy peaks are not important as carriers of speech information⁶. Most of the speech information is carried by the zero-energy crossover points of the wave. This implies that the form of the speech wave is not inherently important, and that liberties can be taken with the speech wave for a hearing aid. Gregory and Wallace² concluded that it should be possible to prevent overloading while retaining the necessary information, by using suitable peak clipping in hearing aids. Amplitude clipping was, however, found (by ourselves and others) to produce unpleasantly distorted sounds, and the Fourier components generated by sharp clipping give masking tones which reduced intelligibility. "Rounding" the flat tops

of the clipped waves with a high cut filter gives some improvement, but removes information since some of the generated components are in the speech frequency band. Alternatives, such as frequency transposition, were also attempted but were abandoned for various reasons³. Amplitude compression (AGC) is standard practice for broadcasting and recording. It is only suitable for hearing aids if the "attack time" is made short enough to limit individual energy peaks (of the order of hundreds of microseconds) and so minimise peak overloading. The "release time" must also be made short enough to preserve information of consonants following vowels without causing sound level fluctuations ("breathing")⁷.

Villchur used a 2-channel amplitude compressor with an attack time of "less than 1 ms" and a release time of 2000 dB s⁻¹. He found improvements of syllable recognition of from 22% to 160% in quiet and 10% to 230% in a "cocktail party" situation. "Improvement was defined as

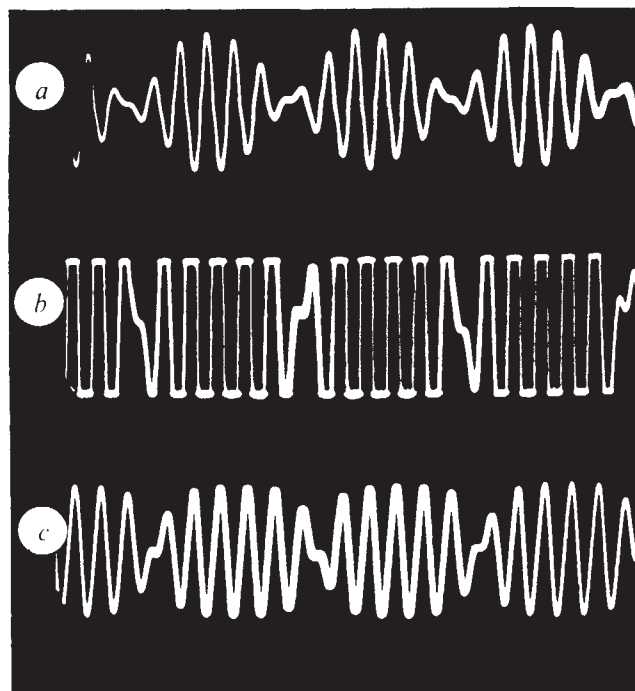
$$100 \left(\frac{\text{score with processing} - \text{score without}}{\text{score without processing}} \right)$$

An alternative and possibly complementary approach is instantaneous amplitude clipping, but without the squaring and generation of cross modulation products in the speech band associated with peak clipping. This has for a long time seemed impossible; but it can now be achieved.

The solution we have adopted is to generate the inevitable cross-modulation products outside the speech band. This is done by amplitude modulating a high frequency carrier with the speech, and clipping the carrier^{8,9}. The cross-modulation products are now multiples of the chosen carrier frequency. By choosing a suitable carrier frequency (50 kHz–100 kHz) the harmonics and cross-modulation products generated by the clipping are widely separated from the speech band, and so can be removed. The result is instantaneous limitation of the audio peaks without flattening or appreciable intermodulation product distortion.

Considering first normal audio frequency peak clipping, any wave exceeding the clipping level will be flattened. For two

Fig. 1 Unprocessed and processed wave forms, from "beating" a pair of AF sine waves. *a*, The unprocessed wave. *b*, Processed by audio frequency peak clipping. *c*, Processed by the carrier clipping system.



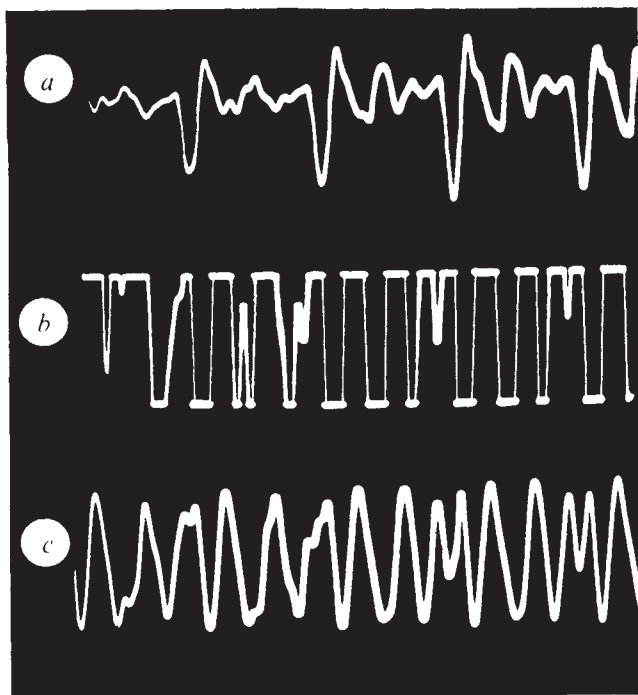


Fig. 2 Unprocessed and processed wave forms of speech. *a*, Unprocessed speech wave. *b*, Processed by audio frequency peak clipping. *c*, Processed by the carrier clipping system. It will be seen that the carrier clipping system retains the wave form while limiting the amplitude. In all cases the peak amplitudes are equal though the small signal amplification is increased in the *b* and *c* waves. The wave form is destroyed with audio frequency clipping (*b* waves) while the wave form is retained by the carrier clipping (*c* waves).

equal amplitude sine waves, with frequencies f_1 and f_2 respectively, the intermodulation products will include

$$(f_1 + f_2)/2 + n(f_1 - f_2)/2 \\ \text{for } n = 3, 5 \dots$$

There will also be another overlapping series of products centred on harmonics of f_1 and f_2

$$m(f_1 + f_2)/2 + nm(f_1 - f_2)/2 \\ \text{where } n = 1, 2, 3 \dots \\ m = 2, 3, 4 \dots$$

With a complex speech wave, there will be corresponding cross-modulation products for each component frequency and its Fourier terms. It is indeed remarkable that audio frequency clipped speech is at all intelligible.

Consider now a single side band suppressed carrier (s.s.b.) signal. If this is clipped, much the same products are produced as in the audio frequency case just considered; but now the intermodulation products and harmonics are widely separated from the signal.

Taking signals $F_1 = 1$ kHz and $F_2 = 2$ kHz and a carrier frequency of 50 kHz then f_1 and f_2 will be 51 and 52 kHz respectively. The modulation products associated with m and n values will be centred on integral multiples of the carrier, 50 kHz. So they are now widely separated from f_1 and f_2 and can be filtered out. The clipped s.s.b. signal is now heterodyned back to the original audio frequencies. The frequencies left will be F_1 and F_2 .

Harmonic frequencies produced by the clipping are lost to the audio band. The clipped waves are not flat topped; but are limited in amplitude with virtually no harmonic distortion. The wave forms are shown in Figs 1 and 2.

Children and adults suffering sensorineural loss as diagnosed clinically have been tested with carefully calibrated equipment in the laboratory. The children attend the Partially Hearing

Unit of Hengrove School, Bristol. Some of the adults were patients at the Bristol Royal Infirmary. Normal subjects were also tested, with added white noise to simulate sensorineural deafness. Comparison was made between three types of speech processing. Speech from pre-recorded test tapes was processed by each of three systems and fed into the subject's ears by headphones. The three processing systems were: (1) linear amplification; (2) audio frequency peak clipping; (3) high frequency carrier clipping (HFCC). In all cases the audio frequency band was restricted to 400 Hz–2.5 kHz, and 16 dB of clipping was used both for audio frequency peak clipping and high frequency peak clipping, that is, the signal was amplified by 16 dB then reduced to the original level by clipping. The apparatus was set up by using a 900 kHz pure tone to equate the output levels for the three systems. The output from the tape recorder was adjusted so that the peak levels of the test words coincided with the level of the calibration tone. The amplification for each of the patients was adjusted for about fifty per cent recognition, using Fry's single word and sentence test tapes. The level for the normal subjects was set so that the calibration tone was 80 dB above the average pure tone threshold, and broad band masking noise of 50 dB (for word tests) or 60 dB (for sentences) was added, after signal processing to simulate sensorineural deafness. Each test lasted about one hour and included about 350 individual words, or 175 test sentences.

The results are summarised in Fig. 3. Most subjects showed an improvement of word recognition with HFCC. This varied from +145% down to -35%.

The scores are shown as

$$100 \left(\frac{\text{word score with HFCC} - \text{word score with linear processing}}{\text{word score with linear processing}} \right)$$

This is the same method of data presentation as used by Villchur.

The most conspicuous difference between the groups of subjects was that the variability was much greater for those with impaired hearing than for normal subjects with noise masking. A similar result was obtained with sentence tests (Fig. 4). Peak clipping was less effective than HFCC.

These results were obtained without practice. No consistent learning effect was observed within a test. An attempt will be made to measure any learning effect which does occur over a timescale of several weeks.

Speech which is amplitude limited in this way sounds almost normal. With equated peak amplitudes it sounds considerably (up to about 10 dB) louder. Intelligibility is increased. Its main advantage in nerve deafness is its ability to amplify the information-bearing features of the speech wave while limiting the peak energies to a safe value. This reduces or removes intermodulation products generated by overloading the mechanism of the middle ear, and protects the cochlea from unnecessary damage.

Six subjects (2 deaf, 4 normal with noise masking) were tested with amplitude compression using the same limiting ratio, with an attack time of 0.25 ms and a release time of 25 ms. The results were comparable to those of Villchur⁷.

For a practical hearing aid, the entire dynamic range of sounds reaching the aid should be considered and the output must remain tolerable in overload conditions. Special consideration should be paid to the energy and frequency ranges of sounds important to the user, especially speech. For normal ears, recognition of speech is about 50% at 20 dB above pure tone threshold. The normal dynamic range of speech is about 60 dB, with a mean of about 40 dB. The upper mean limit considered safe in industry is 90–100 dB and distortion for speech becomes significant at peak energies of about 120 dB. This implies that for the normal ear there is a very small, if any, margin for linear amplification of speech before risk of damage. This applies also to sensorineural deafness, with perhaps more risk of further damage to the impaired cochlea. These

figures imply that an aid suitable for sensorineural deafness should reduce the dynamic range of speech proportionately to the rise in pure tone threshold. This could require as much as 80 dB compression which is difficult to achieve by normal (a.g.c.) compression. For the HFCC system 80 dB compression is possible but gives considerable distortion. A hybrid system could well be adopted, with say 40 dB slow acting a.g.c. compression and 40 dB instantaneous HFCC peak limiting.

According to the prevailing signal level, the HFCC system operates in three modes: (1) for low intensities it is a linear amplifier; (2) for intermediate intensities it gives linear amplification to the low energy components while limiting the high energies; (3) very high signal energies will overload early stages of the system, producing harmonic components which will however be limited in amplitude. We are primarily concerned with the middle energy range, where the aid is limiting without significant distortion. The increase in intelligibility we have found was for peak energies well within the dynamic range of the ear. Where high energies are required the value of the system can be greater—to the point where speech can be

Fig. 3 Percentage improvement of word identification with high frequency carrier clipping. *A*, Pupils at Hengrove Partial Hearing Unit; *B*, patients at Bristol Royal Infirmary; *C*, other adults with impaired hearing; *D*, control group; unimpaired hearing, white noise masking.

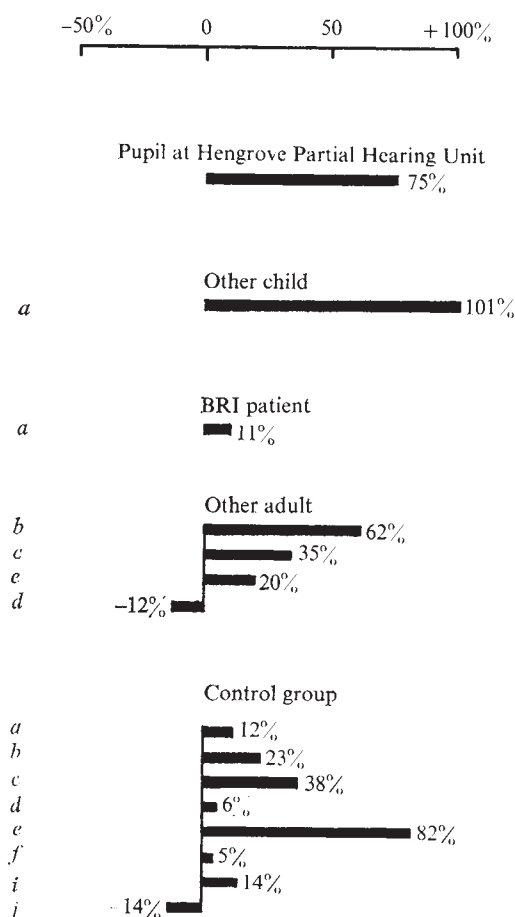
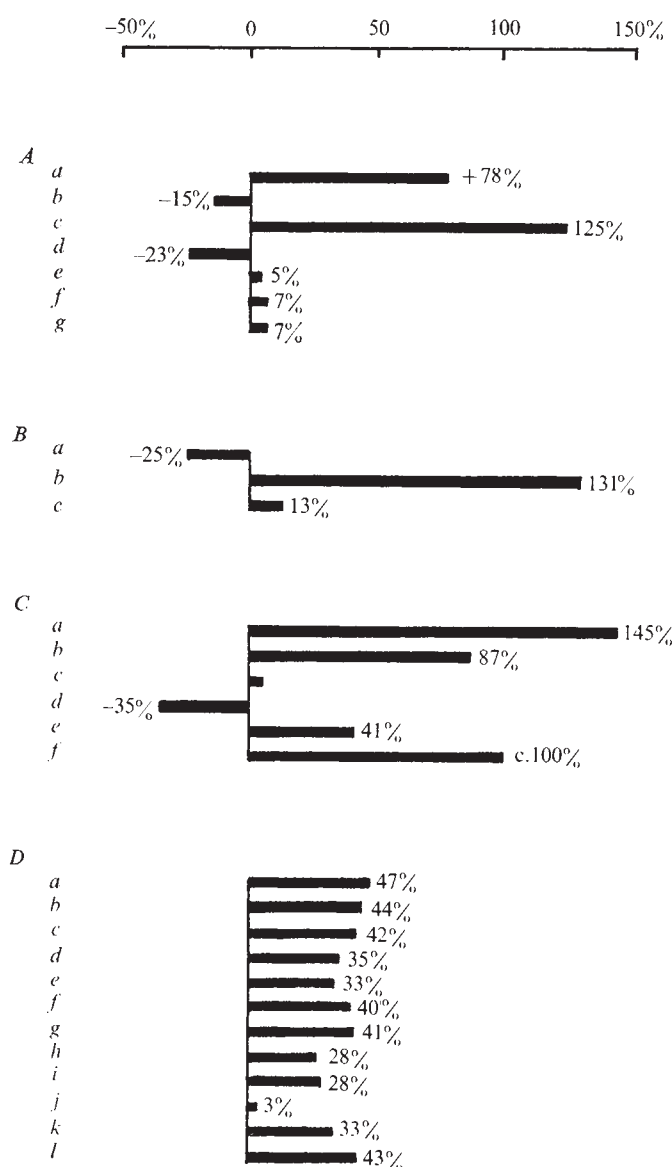


Fig. 4 Percentage improvement of sentence identification with high frequency carrier clipping.

provided by the limiting system but only with extreme discomfort or not at all by normal linear amplification. Very severely handicapped children will, however, require training to accept speech without concomitant lip reading even if the signal is adequate after processing.

Although a few years ago the complexity required would have made a device such as this impracticable, recent advances in electronic circuits and falling cost by no means rule it out as a practical hearing aid. The system has a further advantage over conventional aids: "howl-round" restricts their usable amplification, especially for children whose ear moulds tend to fit loosely. HFCC limits the howl-round energy to a safe, and not unpleasant, limit. We are working on fail-safe methods of avoiding this acoustic disaster.

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Right hemispheric sensitivity for the McCollough effect

THE mammalian cerebral cortex is divided into two hemispheres and it seems in man that each half specialises in a different kind of information processing. Linguistic skills are, for the most part, localised in the left hemisphere while the right may dominate in visual/perceptual tasks¹⁻⁴.

This division of labour has important theoretical and clinical implications but its basis is not understood. However, I have found that pink McCollough effect hues, perceived by subjects after exposure to a green square-wave grating, are reported to be stronger in the left visual field even when initially processed by the right cerebral hemisphere. Since the McCollough effect seems to be a process related to the orientation specific neurones of the striate cortex⁵, this suggests a specialisation for visual perception in the right cerebral hemisphere which may include the earliest visual areas.

Neurones in the striate cortex (area 17) of the primate occipital lobe are the first cortical units to receive retinal information. Electrical recordings from these cells indicate that each may respond only to a specific pattern of light and dark. A cortical neurone, for example, might be sensitive to lines of a certain orientation, width, colour and/or which move in a specific direction⁶. It has been postulated that the superiority of the right hemisphere in some visual tasks, involving orientation³ and brightness judgments⁷, could be due to hemispherical differences between the striate areas^{1,6,7}.

A direct test of the hypothesis that the differentiation in hemispherical information extends to the primary input areas of the cortex is difficult. Electrode recordings from human cortical neurones give limited information, but psychophysical techniques are available to tap these cortical populations⁸, and can be used to exploit the phenomenon known as the McCollough effect⁹. If a subject views vertically oriented green and black bars (Fig. 1a) for several minutes, a similar vertical black and white grating will seem pink. If the orientation or bar width of the grating are varied, this complementary after-effect will weaken. These hues can be made contingent on orientation, width (spatial frequency) or motion which correspond to the selectivities of striate neurones⁹⁻¹¹.

These pattern contingent hues are best explained by a lowered sensitivity in cortical units tuned to both colour and the spatial configuration of the adaptation stimulus^{5,12}. For example, exposure to green vertical lines would adapt out neurones responsive to green vertical. The long duration of the effect suggests that it may be related to learning or memory^{13,14}.

Furthermore, the McCollough effect is orientation and movement selective and must not occur below the primary cortex. Second, the effect does not easily transfer from one eye to the other^{9,12,15-17}. This suggests an early striate locus as this area seems to be more monocular than later cortical regions¹⁸⁻²⁰.

I have tested the strength of the McCollough effect in the left compared with the right cortical hemisphere. Because of the anatomy of the visual pathways, stimuli presented to the left of a fixation point are transmitted to the right hemisphere and those on the right reach the left hemisphere. Thus subjects were exposed to a large green and black vertical 7.5 cycle deg⁻¹ of visual angle, square wave grating (Fig. 1a) over which they freely scanned for

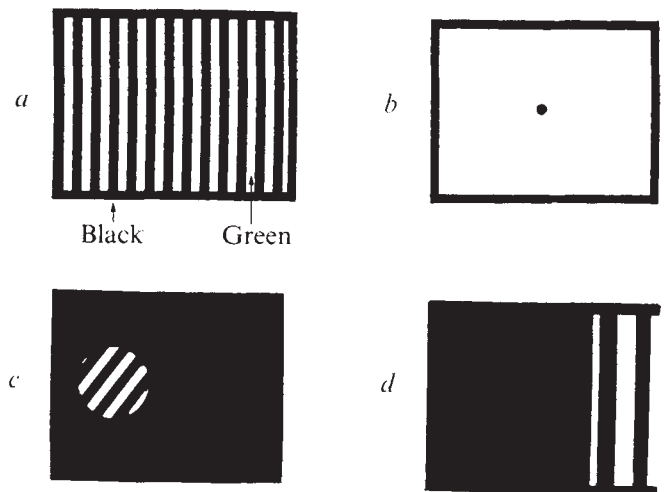


Fig. 1 Descriptions and schematic examples of stimuli. Scale does not exactly correspond with that of the actual slides. *a*, Green vertical adaptation grating (coloured filter Kodak Wratten 53, contrast ratio = 0.97, mean luminance = 19 cd m⁻², visual angle of slide = 4.5° vertically by 8.25° horizontally, spatial frequency = 7.5 cycles deg⁻¹). With green filter removed, slide served as achromatic test standard (see text). Luminance and contrast were constant for all slides in the experiment. *b*, Fixation point, presented for a 500-ms period before each test patch. *c*, Achromatic 30° test patch for right hemisphere (left visual field). Test patch series orientations were 0°, 10°, 20°, 30°, 40° and 50° off vertical. Grating diameter = 2.75° with centre 2.1° from fixation. Total series consisted of 12 slides, 6 with patch to the left of fixation and 6 to the right. *d*, Achromatic +1 octave spatial frequency test patch for left hemisphere (right visual field). Test patch series spatial frequencies varied -2, -1, 0 (7.5 cycles deg⁻¹), +1 and +2 octaves from 7.5 cycles deg⁻¹ of visual angle. Grating patches = 4.5° vertically by 2° horizontally with their innermost edge 1.1° from fixation. Total series consisted of ten slides, five with patches to the left of fixation and five to the right.

250 s. The scanning during adaptation was to reduce the formation of patterned negative afterimages. The adaptation period should produce a pink aftereffect in both visual fields. Small black and white test gratings were then presented for 90 ms to the left or right of fixation (Fig. 1b). These conditions prevented eye movements and assured initial stimulus presentation to the appropriate hemisphere. The strength of the hues was determined as the target gratings varied in spatial frequency and orientation.

It might be expected that if the right hemisphere is perceptually more sensitive or more efficient in some kind of perceptual learning process than the left, the complementary hues would be stronger with the test stimuli presented to the left visual field. The basic idea is to test cortical laterality of function with an after-effect that seems to be produced at the striate cortex.

Three right-handed subjects compared the strength of the McCollough hues in both hemispheres as test patch orientation varied from vertical (0°) in clockwise steps of 10° up to a 50° orientation. (Fig. 1c). After viewing a black and white version (Fig. 1a) of the adaptation grid, the pink perceived on this grid was assigned a value of 10. They were then to rate the hue of the test patches (Fig. 1c) presented to the left or right of fixation on the basis of this "10" value.

Each subject was adapted and tested using the left eye, right eye or both on separate days. Each eye condition was run twice for a total of six sessions with order counter-balanced. On each day the test and adaptation sequence was run five times. After each adaptation period (see text) the test series (Fig. 1c) was run twice with order counter-balanced between and within trials. Eye conditions were not significant and collapsed so that each patch received 60 ratings. The data were transformed by dividing a

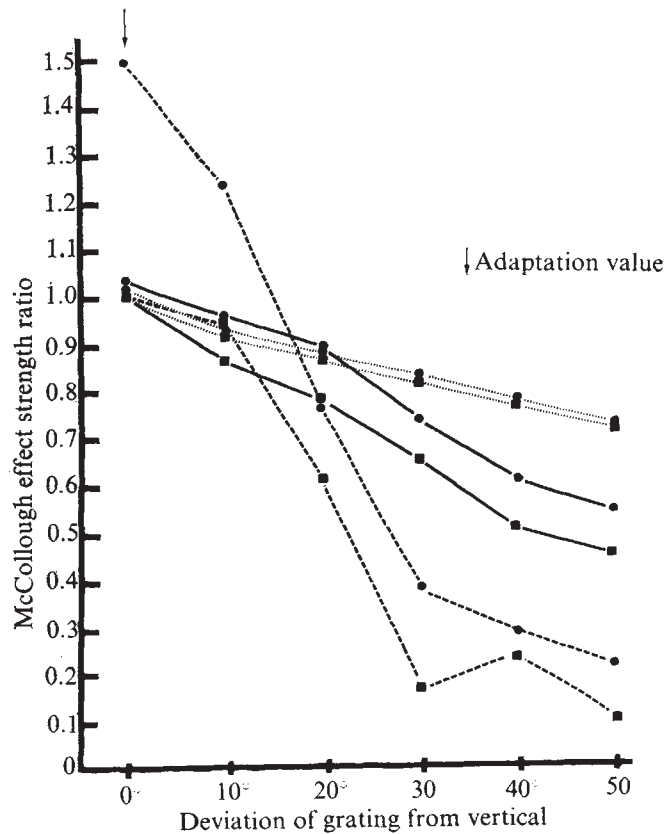


Fig. 2 McCollough effect strength ratios for subjects GL, WE and WH as test grating orientation varied from vertical (0°) as a function of initial cortical hemisphere of stimulus presentation. The vertical orientation used for adaptation is indicated with an arrow. Each point represents 60 ratings (see text). $\bullet$ — $\bullet$: GL, right hemisphere; $\blacksquare$ — $\blacksquare$: GL, left hemisphere; $\circ$ — $\circ$: WE, right hemisphere; $\square$ — $\square$: WE, left hemisphere; $\bullet$ — $\bullet$: WH, right hemisphere; $\blacksquare$ — $\blacksquare$: WH, left hemisphere.

subject's mean for a particular test orientation and cortical hemisphere by that subject's value for the left hemisphere (right visual field) at 0° (vertical) which was the adaptation orientation. This in no way altered the results of statistical analyses for each subject but only served for convenient graphic representation. For comparison, the untransformed left hemisphere 0° mean ratings for GL, WE and WH were 9.05, 8.27 and 0.95 respectively.

Three subjects were similarly run except that the test patch (Fig. 1d) varied ± 2 octaves in spatial frequency from the adaptation value of 7.5 cycles deg^{-1} . Their data were similarly transformed by dividing the subject's mean for a particular spatial frequency and cortical hemisphere by that subject's value for the left hemisphere (right visual field at 0 octaves; 7.5 cycles deg^{-1}). Again, for comparison, the untransformed left hemisphere 0 octave mean ratings for JC, WH and GL were 4.72, 3.72 and 6.13 respectively.

The ratios for the orientation conditions are presented in Fig. 2. For each subject, the strength of the pink McCollough effect significantly decreased as orientation varied off vertical (GL, WH, WE; $P < 0.001$). Right hemisphere hues were consistently stronger than the left (GL, $P < 0.04$, WH, $P < 0.003$; WE, $P < 0.001$). The spatial frequency data in Fig. 3 are similar. The after-effect decreased as spatial frequency varied from 7.5 cycles deg^{-1} (WH, GL, JC: $P < 0.001$), and the right hemisphere hues were strongest (WH, $P < 0.004$; GL, $P < 0.001$; JC, $P < 0.02$).

The drop in the pink McCollough effect as orientation and spatial frequency varied off the adaptation value is typical and indicates a cortical locus^{9,10,12}. There are three possible explanations for the stronger right hemisphere hues: (1) degradation of information quality during trans-

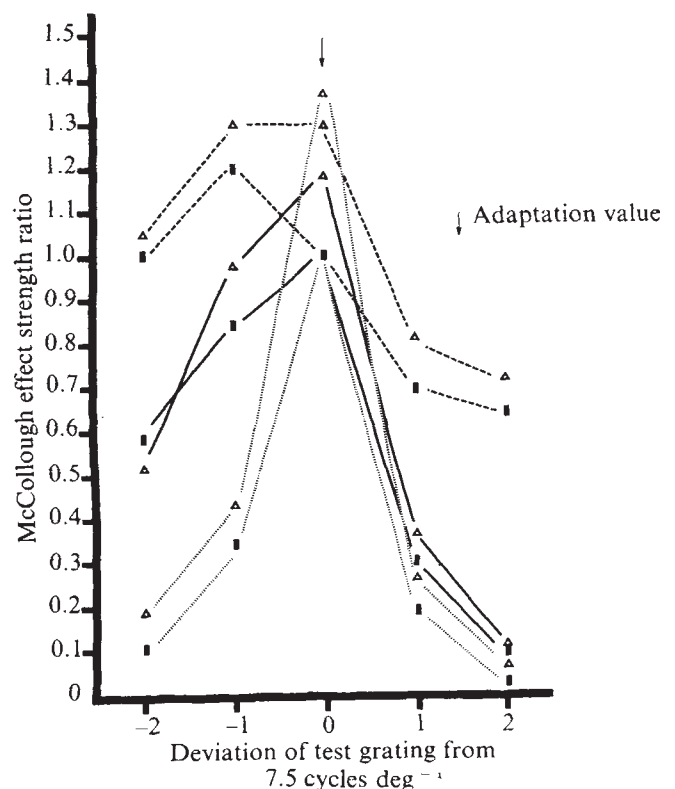
mission between the hemispheres to appropriate processing centres, (2) greater attentional activation of one hemisphere depending on task requirements and (3) the right hemisphere superiority in visual perception is due to sensitivity on processing differences possibly down to area 17 (ref. 21).

The first theory implies that the rating procedure is a function of the right hemisphere and that information from the left is degraded as it is transferred to right for judgement. One might predict, however, that such a process would increase the variability of the left hemisphere scores but no significant trend for larger left hemisphere variances were found for any subject (Bartlett-Box F , $P > 0.10$). However, a constant decrement is possible and cannot be totally eliminated.

The second model²² assumes that if a cognitive strategy proper to one cortical hemisphere is adopted, attention is biased to the contralateral visual fields. If hues ratings activated the right hemisphere to a greater extent, this would explain the present data. Attentional biases in this model, however, are generated in favour of the left hemisphere if there was an expectancy of a verbal processing which the present experiment did include. Thus, the present data are contrary to the original model. Also, experiments designed to reduce any attentional biases still demonstrate hemispherical effects²¹.

The superiority of the right hemisphere in visual tasks, as postulated by the third theory, might be due to enhanced contrast of the stimulus or a longer duration of the stimulus trace⁶. Either one of these postulated improvements in visibility operating on the adaptation and test stimuli could produce stronger McCollough effect hues. Also, if the

Fig. 3 McCollough effect strength ratios for subjects JC, WH and GL as test grating spatial frequency deviated from 7.5 cycles deg^{-1} of visual angle (0 octaves) as a function of cortical hemisphere of initial stimulus presentation. The adaptation frequency is indicated with an arrow. Each point represents 60 ratings. $\triangle$ — $\triangle$: JC, right hemisphere; $\blacksquare$ — $\blacksquare$: JC, left hemisphere; $\triangle$ — $\triangle$: WH, right hemisphere; $\blacksquare$ — $\blacksquare$: WH, left hemisphere; $\triangle$ — $\triangle$: GL, right hemisphere; $\blacksquare$ — $\blacksquare$: GL, left hemisphere.



orientation specific adaptation phenomenon with its hypothesised loss in sensitivity in cortical units is a neural modification akin to memory, it might be more efficiently and effectively implemented for perceptual tasks in the right hemisphere given the latter's predominance in such processing.

Information transmission problems and attentional shifts are possible but the variation of early cortical sensitivity seems to be the more parsimonious and interesting potentiality at this stage of analysis. If the latter is true, whether these differences are developmental in nature or innate is quite fascinating. In primates, the striate populations are stimulus selective at birth but modifiable by experience²⁰. Any interaction between visual cortical perceptual development and the laterality of information processing is relatively unexplored.

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Effect of monocular deprivation on binocular neurones in the owl's visual Wulst

DESPITE the two hundred million years during which their evolutionary history was different from mammals, birds possess a central visual apparatus with surprising functional similarities to the striate cortex of cats and monkeys¹. The visual Wulst² of the owl contains neurones which can be binocularly activated and which show precise selectivity for the orientation, direction of movement and binocular disparity of moving straight line contours¹, all characteristic properties of single neurones recorded from the striate cortex of cats and monkeys^{3,4}. We were interested to determine whether binocular neurones in the owl's Wulst are also sensitive to visual experience in the neonatal period since another important characteristic of binocular neural connections in cat and monkey visual cortex is their extreme sensitivity to monocular deprivation during the critical period^{5,6}. The preliminary observations we present here, on young, monocularly-deprived owls, suggest that the functional parallel between the mammalian striate cortex and the avian Wulst extends to the phenomenon of plasticity as well.

Data were obtained from 8 barn owls (*Tyto alba*) which had been hand-raised indoors. Six had relatively normal early visual experience in the laboratory and provided control data for this study in addition to detailed data presented elsewhere¹. The ages of the 6 normal owls ranged from 1 to 7 years. Two owls had had the right eyelid sutured under halothane anaesthesia (1-1.5% in 50% O₂: 50% N₂O) at the ages of 11 d, at the time of normal eye opening (Owl T) and 29 d (Owl D, both of whose eyes had opened at 7 d) respectively. Both monocularly lid-sutured owlets seemed unaffected by the procedure if we compared their behavioural development with normal owlets. For example, the typical, side-by-side, scanning head movements were first used by Owls D and T to locate visual targets at the same age as normal owlets.

Single unit recording was carried out with tungsten-in-glass extracellular microelectrodes⁷ from a closed chamber over the lateral margin of the visual Wulst (representing the visual field within 10° of the vertical meridian¹) under ketamine anaesthesia (12 mg/kg intramuscular).

All birds, including the two monocularly-deprived owls, were fully grown (average 550 g) and fully fledged at the time of recording. Owls T and D were 3 and 4 months old, respectively. Corneas were protected with plastic contact lenses (radius of curvature, 6.5 mm). Visual stimuli were presented on a rear projection screen 57 cm from the nodal point of the eye by a custom-built projector which allowed joystick control of position, orientation and size^{8,9}. Retinal landmarks were projected ophthalmoscopically on to the screen and the vertical meridian and visual axes established indirectly, as is done in the cat¹⁰, from the position of the optic nerve head, which is overlaid in birds by the easily distinguished pecten oculi¹. Variable biprisms set before each eye made it possible to control binocular retinal disparity¹.

As described in detail elsewhere¹, the Wulst of the normal owls contained a high proportion of binocularly activated neurones, including a significant fraction which were driven only by simultaneous binocular stimulation with targets at the appropriate binocular disparity (Fig. 1). These latter neurones, which show functional similarities to the binocular depth cells recorded in the extrastriate visual cortex of monkey¹¹, were found only in the more superficial layers of the Wulst, around 1 mm from the surface.

In marked contrast, both monocularly deprived owls lacked binocularly activated neurones (Fig. 1). In the superficial layers, where we encountered the cells with an absolute requirement for binocular stimulation in normal owls, many cells had spontaneous activity but were unresponsive to any visual stimulation we could devise. In the deeper layers where we recorded the majority of neurones, there were uninterrupted sequences of cells with brisk responsiveness and receptive fields which could be sharply defined with moving edges of the appropriate orientation and direction of movement. These sequences were therefore like those through the Wulst of normal owls, except that the receptive fields could be defined only for one eye, which was in most cases the non-deprived eye. We also recorded a number of neurones, particularly from Owl T, which had receptive fields in the deprived eye only. All of these cells had poorly defined preferences for stimulus orientation but were briskly responsive, a strong indication that there was a functional input to the Wulst from the deprived eye.

Another striking difference between normal and monocularly-deprived owls was the presence of a marked strabismus in the latter. As measured by the projection of the tip of the inferior limb of the pecten oculi, where it meets the optic nerve head, eye alignment is extraordinarily precise in normal owls. The pecten tips of each eye are in the same horizontal plane and are separated by 56° (s.d. ± 0.9°, N=6) when projected into space through the nodal points.

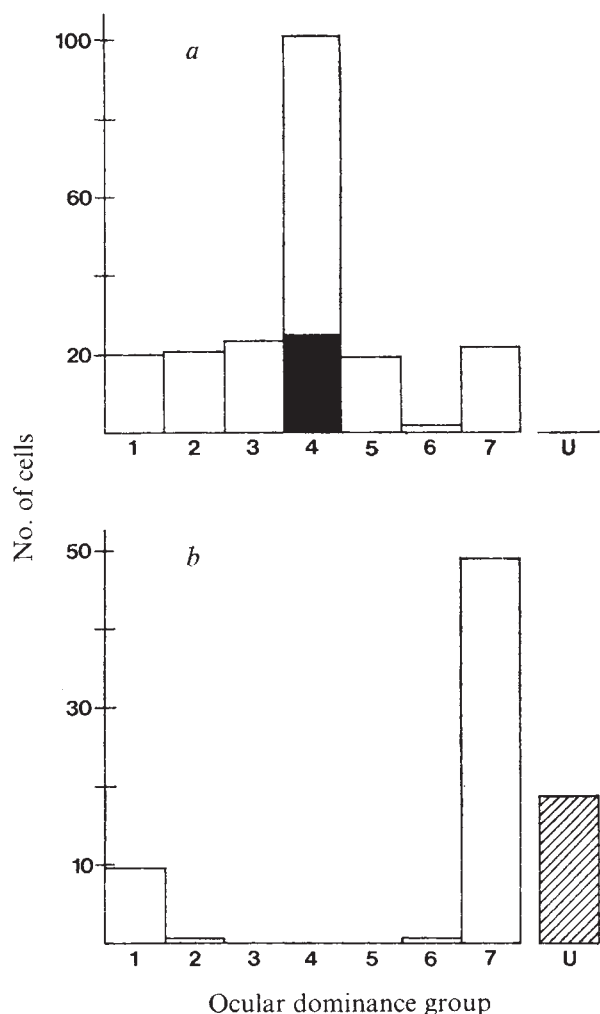


Fig. 1 Ocular dominance histograms obtained from the visual Wulst of 6 normal owls (*a*) and two owllets each of whose right eye had been closed for 2–3 months (*b*). All data shown were obtained from electrode tracks in the left hemisphere. A total of 10 electrode tracks provided the data in *a* and two tracks (one in each of the monocularly deprived owls, T and D) provided the data in *b*. A third electrode track in the right hemisphere of Owl T yielded seven visually unresponsive cells and seventeen driven exclusively by the contralateral (non-deprived) eye. These data are not included in the figure to avoid possible confusion caused by the reverse order of ocular dominance numbers which would apply to the opposite hemisphere. Single neurones were assigned an ocular dominance category according to the criterion of Hubel and Wiesel³, where group 1 represents cells driven exclusively by the contralateral eye, group 4, cells driven equally well by both eyes, and group 7, cells driven exclusively by the ipsilateral eye. The group 4 cells in the filled portion of histogram *a* could be driven only by simultaneous presentation of an oriented target to both eyes at a certain binocular disparity and could not be driven independently from either eye. The shaded area of histogram *b*, marked "U" indicates cells which were unresponsive to visual stimulation. Both the filled cells in *a* and the shaded cells in *b* were recorded in the superficial layers of the Wulst.

There was clear vertical and horizontal misalignment in both owls T and D whose pecten tips were separated by 66° and 96° respectively. Although strabismus is a feature of monocularly deprived kittens¹² it was very surprising to us in this case because of the absence of eye movements greater than a degree or two in adult owls^{1,13}. Perhaps eye movements are more prominent in the owl's early development when they could allow the final precise alignment to be achieved with visual feedback.

The present findings strongly suggest that binocular visual experience is as important for the development of the avian visual Wulst as it is for mammalian visual cortex.

We postulate that a requirement for binocular input during development may be a universal feature of binocular visual systems which have evolved to subserve the subtle disparity-detection task required for stereopsis⁸. We do not yet know how age dependent are the effects of monocular deprivation in the owl, nor do we know whether binocular neurones can be found in the Wulst before visual experience of any kind.

Finally, it should be pointed out that the striking functional similarities between visual Wulst and visual cortex exist in spite of a number of differences between their respective visual pathways. These organisational differences might be exploited for a better understanding of both systems. For example, since the input and output fibre pathways of the Wulst are separate, one could test the role played in monocular deprivation by the output pathway from the Wulst back to its thalamic input, an impossible experiment in mammals, where both input and output fibre pathways run together.

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Monocular deprivation in kittens impairs the spatial resolution of geniculate neurones

SELECTIVE exposure of kittens to periodic gratings during the early period of life produces a depression of neural responses to gratings of the same or similar spatial frequency¹. Recently we have found that in these kittens also the behavioural contrast sensitivity is depressed at the spatial frequency of exposure (A.F. and L.M., in preparation).

Plastic changes in the functional properties of the visual system caused by restricted visual experience during the critical period have been described mainly at cortical level, although for instance total deprivation of visual experience in one eye can induce anatomical changes in the lateral geniculate (LGN) layers². The early exposure to periodic gratings, however, is found to affect the spatial frequency characteristics not only at cortical level but also at the level of the LGN³. This suggested to us that deprivation of visual stimuli might also effect the spatial frequency responses of geniculate neurones. We report here evidence suggesting that monocular deprivation impairs the resolution of the neurones in the corresponding layers of the geniculate body. A similar result has recently been obtained by Ikeda and Wright⁴ who have reported that in strabismic kittens the resolution of geniculate cells is impaired.

Experiments were performed in 4 kittens in the first week of their life. Using halothane anaesthesia we sutured the

eyelids of one eye (in 3 kittens the right eye, in one kitten the left eye). In addition to suturing the eyelids we drew the nictitating membrane across the cornea and sutured it to the conjunctive along the upper lid.

The recording experiments were performed when the kittens were at least 3 months old. On the day of the experiment the cat was anaesthetised with halothane, and endotracheally intubated. Two small openings were made over the projections either of the lateral geniculate bodies or of the optic tracts. Afterwards the animal was immobilised with curare and anaesthesia was continued with nitrous oxide (80% NO₂ + 20% O₂). Animals' pupils were dilated with atropine, contact lenses with artificial pupils 4 mm in diameter were applied to both eyes and refraction was carefully corrected with additional lenses.

The action potentials of geniculate neurones or optic tract fibres were recorded extracellularly with tungsten microelectrodes and receptive fields were mapped by projecting the images of bright spots or bars on to a tangent screen 57 cm from the cat's eye. For each isolated neurone we obtained the average responses to 20 stimulus repetitions using drifting square-wave gratings of high contrast and various spatial frequencies (for details of the method see ref. 4).

We have done 9 penetrations in the LGN and one in the optic tract recording from 121 neurones. For each neurone we measured the "visual acuity", defined here as the highest spatial frequency of the drifting grating that produced a just detectable modulation of the rate of discharge in the averaged responses. The receptive fields of the neurones recorded in each penetration were at least partially superimposed and fell within a restricted area of the visual field. In some penetrations this area was within the area centralis, in others it was eccentric to it, but never more than about 20°. We have found that the average visual acuity of neurones activated by the deprived eye is approximately one half that of the neurones activated by the normal eye and projecting to the same part of the visual field. In Fig. 1 we present the visual acuity of neurones for a penetration through the LGN ipsilateral to the deprived eye (*a*) and for a penetration through the LGN contralateral to the deprived eye (*b*). It is clear that going from a layer to the next (from filled circles to open circles, or *vice versa*) brings about an abrupt change in the average visual acuity.

The results obtained in all the penetrations are summarised in Table 1. For each penetration, we reported the number, *N*, the mean visual acuity, *m*, and the standard

Table 1 Acuity of cells in normal and deprived eyes of monocularly-deprived kittens

Penetration	Cat	Normal eye			Deprived eye		
		<i>N</i>	<i>m</i>	s.d.	<i>N</i>	<i>m</i>	s.d.
1	1	9	2.1	0.4	8	0.9	0.3
2		4	2.0	0.4	7	0.8	0.3
3	2	4	2.1	0.8	8	1.1	0.9
4		4	1.7	0.4	6	0.5	0.3
5		9	2.0	0.5	11	0.8	0.3
6		7	1.9	0.6	6	0.8	0.2
7	3	4	3.1	0.2	5	2.8	1.1
8		8	1.5	0.3	10	0.8	0.3

deviation of cells activated by the normal eye (left columns) or by the deprived eye (right columns) and located in either of the two upper layers of the LGN (layer A, contralateral eye, and A₁, ipsilateral eye). Penetrations 1, 5 and 6 were in the LGN ipsilateral to the deprived eye, penetrations 2, 3, 4 and 7 on the opposite side. All the cells recorded had receptive fields located within 5° from the centre of the field, except for penetration 4, that projected 10° peripherally. Two penetrations in which we recorded only 4 and 6 neurones (kitten 2 and 4, respectively) have not been included in Table 1. A few neurones located in layer C have also been omitted. In the results we have reported we have not discriminated between X and Y neurones, because we found that the great majority of cells recorded in the deprived layer had lower visual acuity than the neurones in the normal layer.

The data for penetration 8 in Table 1 were obtained recording from optic tract fibres projecting to a restricted area of the visual field. They look very much like the statistics evaluated for similar samples of neurones of the LGN, suggesting that the retinal neurones of the deprived eye might be involved in the loss of visual acuity observed at the LGN level. A similar hypothesis was proposed previously³. Further recordings from retinal fibres would be required to assess this point.

We conclude that visual deprivation of one eye during the early period of life in cat impairs, on the average, the spatial resolution of the neurones in the LGN on to which the deprived eye projects.

Experiments are in progress to investigate whether the plasticity observed in LGN neurones is of cortical origin or is present even at a retinal level, as the one penetration we did in the optic tract could indicate.

Ikeda and Wright³ suggested that the loss of resolution in LGN neurones of cats with surgically induced squint could have a bearing on the human strabismic amblyopia. The similar effect induced in our kittens by deprivation from patterned vision could be at the basis of the amblyopia caused in human subjects by anisometropia (when patterned vision is dramatically impaired in one eye by a large optical defect) or by corneal or lens opacities occurring in one eye in the early years of life. The fact that neural resolution can be affected in the deprived animals is a further warning against the prolonged patching of one eye, a practice still in use in the treatment of squint.

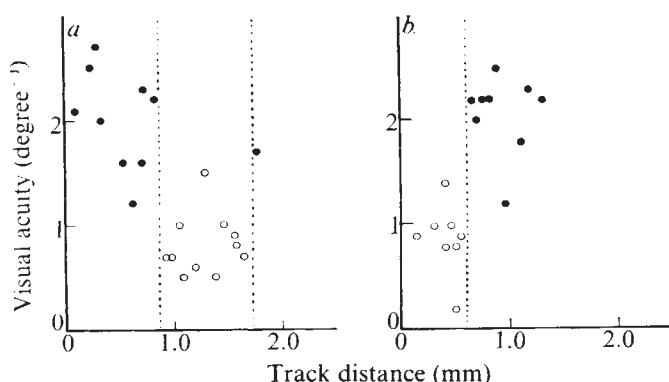
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Fig. 1 Visual acuity of neurones recorded in two penetrations through layers A and A₁ of the LGN of 2 kittens plotted as a function of electrode depth in the LGN. *a*, Penetration ipsilateral to the deprived eye, 4° eccentricity. *b*, Penetration contralateral to the deprived eye, 2° eccentricity. The broken lines indicate the passage from one layer of the LGN to the deeper one, as indicated by a change in the eye that could elicit a response. ●, Open eye; ○, closed eye.



Uptake of soil capillary water by ghost crabs

SEVERAL semiterrestrial crabs, including some ghost crabs (genus *Ocypode*) can extract interstitial water from damp sand¹⁻³. This enables them to offset their high evaporative losses even in habitats lacking surface water. The mechanism involved has remained unknown, although there have been a few observations of how *Gecarcinus lateralis* takes up droplets of water applied to its venter⁴. I have found that uptake of soil water by *Ocypode quadrata*, the common ghost crab of Caribbean and temperate Atlantic North American beaches, is accomplished by a mechanism involving capillary attraction and the production of a substantial vacuum.

Uptake of soil water by terrestrial arthropods requires at least two steps. First, water must be collected by a force exceeding the capillary attraction of the soil spaces. Second, the water must be drawn, past the cuticular exterior, into contact with an ion- and water-transporting epithelium for absorption. These steps have been elucidated only in some small spiders, which can draw up water directly from soil spaces with the sucking mouthparts, against capillary attractions of up to 500 mmHg, and transfer it to the gut for absorption⁵.

In semiterrestrial crabs, which are much larger, the water-collection step has been attributed to tufts of setae located on the bottom parts of the body^{1-3,6}. In *Ocypode quadrata* prominent tufts occupy the bases of the second and third walking legs. The tufts (shown spread apart in Fig. 1) are usually closely apposed and enclose the single posterior aperture to each branchial chamber.

Several experiments indicated that these hydrophilic tufts were essential to the uptake process. Crabs desiccated to 5-10% weight loss, when placed on damp sand, promptly sat down and brought the tufts into intimate contact with subsurface sand by oscillating briefly from side to side. The tufts soon became saturated with water. Water collection, and thus uptake, was prevented if contact of the tufts with sand was prevented by gluing on rubber flaps as a loose cover, or if the capillarity of the tufts was destroyed by matting them with lacquer.

Although the tufts account for water collection, they do not seem to be the site of absorption into the blood. The tufts require cuticular stiffening to penetrate the sand; thick cuticle usually prevents rapid water or ion transport. Also, crabs on sufficiently damp sand bubbled surplus water out around the mouthparts, suggesting that water was transferred to the branchial chambers. When desiccated crabs were placed on sand dampened with seawater containing 0.1% of methylene blue, the tufts did not stain darkly. Within 30 min, however, the gills stained intensely, and within 60 min the mouthparts were moderately stained by the dye solution bubbling out around them. *Ocypode* gills⁷, and portions of *Gecarcinus* gills⁸, have fine structure typical of osmoregulatory rather than gas-exchange membranes. These observations indicate that the absorption step is the function of the gills and that the tufts accomplish only water collection.

The gills and tufts are some distance apart (Fig. 1), and so water must be transferred from the area of collection to the area of absorption by a force exceeding the capillary attraction of the tufts, which itself must exceed the capillary attraction of the soil. Although capillary channels under the carapace can apparently conduct free water to the gills of *Gecarcinus*^{4,6}, it seems unlikely that they could exert the force required to extract soil water. In any case, no such channels communicate with the tufts of *O. quadrata*, and capillarity cannot account for the movement of water to the gills through its relatively large posterior passages. The most

obvious alternative hypothesis, that some sort of wringer action squeezed the water out of the tufts, was eliminated because water could move from tufts to gills without tuft or leg movement.

The remaining hypothesis, that the crabs could generate sufficient vacuum to suck the water from the tufts into the gill chamber, seemed improbable. To test this hypothesis, I drilled through the calcified carapace over each branchial chamber of several crabs (under CO₂ anaesthesia), but not through the underlying membrane, with a high-speed diamond-abrasive dental drill. The highly vascular chamber lining was perforated with a red-hot wire to cauterise it and minimise the otherwise copious bleeding. Cannulae (cutoff 20-gauge hypodermic needles) were fixed with epoxy resin into the holes so that the branchial chambers could be vented or sealed easily. Desiccated crabs with open cannulae were unable to draw up water from damp paper towelling, or even from a thin film of free water. Fourteen such animals lost an average of 0.01% body weight during 8 h on damp sand. Within 4 h of the cannulae being sealed, however, they had gained an average of 6.8% body weight on the same sand, indicating that venting, not surgical trauma, prevented water uptake.

To verify that the uptake of water when cannulae were sealed was indeed due to negative pressures produced in the branchial chambers, I connected the cannulae of three desiccated crabs to a Physiograph recorder equipped with pressure transducers (E & M Instruments). When the crabs were placed on damp sand (5.0-7.5% distilled water) they immediately worked their tufts into the sand. After several minutes, during which the tufts became wetted by a continuous water film, the branchial chamber pressure abruptly fell from ambient to as much as 40 mmHg below ambient, where it remained for a few seconds to several minutes. This was repeated at irregular intervals (Fig. 2).

The maximum suction produced was surprisingly high: in each crab tested it was sufficient to draw up a column of water 55 cm high. This is particularly remarkable in view of the constraints of crustacean structure. The fluttering of the scaphognathite of the first maxilla in a close fitting passageway is adequate to move water through the branchial chambers of aquatic crabs. The movement of air against a large pressure gradient, however, requires much better

Fig. 1 *Ocypode quadrata* with portion of carapace cut away, showing relative positions of structures involved in water uptake. Surplus water may be dumped from the branchial chamber, around the mouthparts, through a passage (not shown) under the antero-lateral carapace.

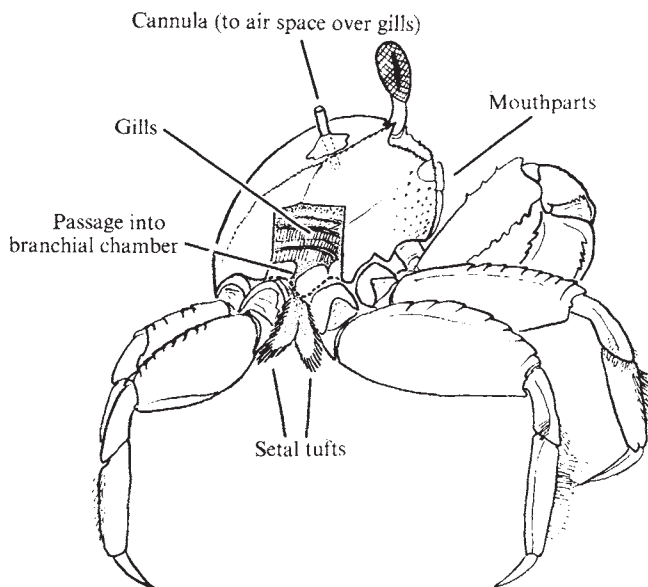
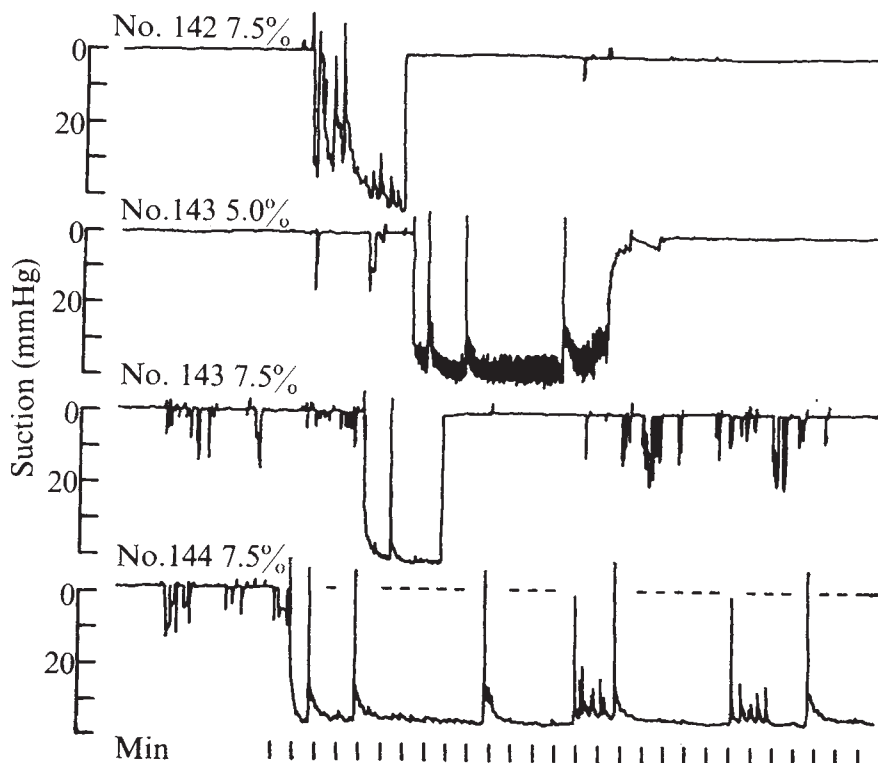


Fig. 2 Representative segments of pressure recordings from branchial chambers of desiccated crabs on damp sand (5–7.5% distilled water). Traced from original recordings.



sealing and the addition of one-way valves to such a pump. The scaphognathite is evidently used in vacuum production by *O. quadrata*, but how it and associated structures have been modified for this purpose is not clear. Also remarkable is the airtight seal formed at the carapace margin, even though fusion of the margin with the irregular area where the legs join the body is precluded by the necessity of shedding the branchial chamber lining at moulting. The seal seems to consist of a close set row of fine setae fringing the carapace margin. These presumably retain a film of water by strong capillarity; the water serves as the gasket.

At the threshold soil-water content for uptake by *O. quadrata* (3–5%), maximum suction produced by the crabs exceeds soil capillarity (the soil-water tension commonly measured by soil scientists) by 2–10 mmHg. The excess suction represents the pressure gradient available to move water from soil spaces through the tufts to the gills. It is interesting that as soil water content drops below 3–5%, capillary attraction, and thus the vacuum required to remove additional water, increase considerably. *Ocypode quadrata* has evidently refined its vacuum mechanism to the limits of practicality.

The water collection and vacuum mechanisms involve only bulk transport of water; consequently their operation is identical whether sand is dampened with distilled water or seawater. The crabs, however, do not take up fluids indiscriminately, but avoid those likely to cause osmotic stress. For example, desiccated crabs given sand dampened with distilled water rapidly regain weight up to their approximate initial weight and then avoid further uptake; crabs given seawater-dampened sand continue to collect water and bubble the surplus from the branchial chamber.

The presence of setal tufts associated with water uptake in several genera of semiterrestrial crabs (*Gecarcinus*^{4,6}, *Cardisoma*⁴, *Ocypode*^{3,4}, *Sesarma* (personal communication from L. Abele), and *Uca* (personal communication from L. Powers)) probably represents convergent evolution stemming from a shared problem. The setal tufts may have served as intake screens during the intertidal phase of terrestrial invasion, preventing fouling of the gills by suspended sediment particles. To exclude particles while passing respiratory water, the setae would have to be closely

spaced, stiff and wettable—attributes which would preadapt them for soil water collection. Well sealed branchial chambers and air pumps may also be regarded as adaptations to intertidal life, facilitating particle exclusion and permitting survival during tidal exposure.

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Colony incompatibility in bacteria

WE report here the demonstration of colony incompatibility between different strains of the same species of various Enterobacteriaceae, using chemotactic migration through semi-solid agar. This phenomenon has hitherto only been demonstrated in the swarming bacteria, *Proteus*, where it is known as the Dienes phenomenon.

Proteus bacteria are unusual in that they readily swarm out from the inoculum point on the surface of most agar media. Despite study by many workers there are several aspects of this swarming that are not understood. We have been considering one of these aspects, namely the behaviour of two swarms as they approach one another. When two different swarming strains of *Proteus* meet on agar there often occurs what has been described as “one of the most peculiar forms that antagonism can assume”¹. This effect, known as the Dienes phenomenon, as well as the swarming of *Proteus*, has been illustrated in a review². Some hours after the two swarming colonies have met and merged, a

demarcation zone about 1 mm wide of sparse bacterial growth and containing many aberrant cells appears^{3,4}. The effect has been of some value in epidemiological studies^{5,6} in determining whether two isolates belong to different strains. Although 30 years have elapsed since the phenomenon was discovered⁷, little progress has been made towards elucidating its basis, nor have comparable forms of colony incompatibility been reported in other bacteria.

Is the phenomenon therefore the result of a form of incompatibility occurring only in *Proteus*, or is it an unusual expression of a more widespread antagonism (or of antagonisms), the unique features being associated with the spectacular swarming characteristic of *Proteus*? Swarming and the Dienes phenomenon are usually demonstrated on a firm agar medium, and on such media other bacteria do not swarm. Bacteria can, however, be induced to migrate rapidly by means of chemotaxis, an effect widespread in microorganisms⁸. Adler⁹ showed that with soft agar media¹⁰ a form of swarming—chemotactic migration through agar—can be produced in *Escherichia coli*. We have now used this soft agar medium to demonstrate colony incompatibility in *Proteus mirabilis*, *E. coli*, and *Salmonella* spp.

Replicated tests were carried out at 37 °C in a water-saturated incubator on four strains of *Proteus mirabilis* (2953, 2960, CW 237 and CW 349, obtained from Mr T. L. Pitt, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT) on firm and soft nutrient agar (Oxoid Nutrient Broth, 1.3%; Difco Bacto-Agar at 1.5% or 0.35%) and firm and soft Tryptone agar (Difco Bacto-Tryptone, 1%; NaCl, 0.5%; Difco Bacto-Agar at 1.5% or 0.35%) in all possible combinations of pairs. Petri dishes were left 2–3 d before use to ensure that the agar was free of drops of surface water which would cause spreading rather than swarming of colonies. On both the firm agar media the colonies spread rapidly across the agar surface, with the occasional pauses of about an hour characteristic of zoned swarming. Inoculation of one strain at one side of the Petri dish and the other strain at the opposite produced, in all but one combination, a clear Dienes line where the swarming colonies met at the centre of the dish. With Petri dish lids removed, under good illumination and against a dark background the demarcation line could be recognised as a zone of less dense growth, and viewed obliquely, one or two ridges could be seen at the surface representing sharp limits to dense growth. Although Dienes reactions were usually obtained within 1 d, the surface ridges often became more pronounced later, so recording was carried out at 2–3 d. Control cultures in which a single strain was inoculated at opposite sides of the Petri dishes showed neither diminished growth nor surface markings where the colonies had met. On both the soft agar media the bacteria migrated from the point of inoculation through the medium as circular bands of steadily increasing radius, as described for *E. coli* by Adler⁹, with subsequent dense growth behind the advancing front. Where two colonies of an identical strain meet at the centre of a Petri dish there is often an area of more sparse bacterial development than elsewhere—possibly the simultaneous arrival of two bands of bacteria leads to rapid nutrient depletion and chemotactic migration out of the area. In consequence of this behaviour in controls, sparse growth where colonies meet is not a reliable indication of incompatibility of soft agar media, and assessment has to be on the basis of surface ridges alone. By this criterion, two combinations of strains (CW 237×CW 349, CM 237×2953) showed clear incompatibility on both firm and soft agar media, three combinations (CW 237×2960, 2960×CW 349, 2960×2953) on firm but not on soft agar, and one combination (CW 349×2953) on soft agar only. Hence all combinations of strains displayed incompatibility, but in some instances it was more readily demonstrated on firm agar and in some with soft.

Tests were carried out with several strains of *E. coli*

and *Salmonella* spp. on soft Tryptone agar. Before experiments strains were passaged through Cragie tubes to ensure motility¹¹. Migration, growth and incompatibility reactions similar to those of *Proteus* on soft agar media were obtained with both *E. coli* and *Salmonella* spp. Some pairs of strains consistently gave reactions and others consistently did not. With *E. coli*, for example, NCTC (National Collection of Type Cultures) 9002 never gave a reaction with NCTC 9001, but both gave reactions with NCTC 9004. It is concluded that colony incompatibility reactions resembling the Dienes effect occur in members of the Enterobacteriaceae other than *Proteus*, and that the previous recognition of the phenomenon in *Proteus* alone was a consequence of the striking form the reaction takes in this genus which is capable of fast swarming on firm agar.

One of us (K.A.B.) intends to investigate the ecological and pathological implications of colony incompatibility in *E. coli* and *Salmonella*, the other (M.J.C.) the ultrastructural, genetic and biochemical basis of the effect in *E. coli* and *Proteus*. It is possible that bacteriophage or bacteriocins are responsible, even though in some instances claims to have excluded one or other of these possibilities have been made¹; on the other hand, some novel form of antagonism may be involved.

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Suppression of malaria infection by oxidant-sensitive host erythrocytes

DEFICIENCY of the red cell enzyme, glucose-6-phosphate dehydrogenase (G-6-PD), predisposes erythrocytes to oxidant-induced haemolysis and is thought to protect humans against severe malaria infection. We have already shown that the malaria parasite exerts an oxidant stress on infected red cells and suggested that premature lysis of malaria infected erythrocytes might occur in G-6-PD deficient humans¹, thus limiting the severity of infection by enforcing the release of immature parasites incapable of propagating the infection².

The geographical coincidence of several types of quantitative deficiency of G-6-PD and endemic malaria suggests that these X-linked enzyme deficiencies have an adaptive advantage in malaria-ridden human populations. Although experimental inoculations of G-6-PD deficient human volunteers with *P. falciparum* have not revealed any difference in the early course of the disease³, both epidemiological^{4,5} and direct⁶ evidence from naturally occurring infections indicates that this enzyme deficiency does protect against fulminant malaria. Vitamin E deficiency confers an oxidant

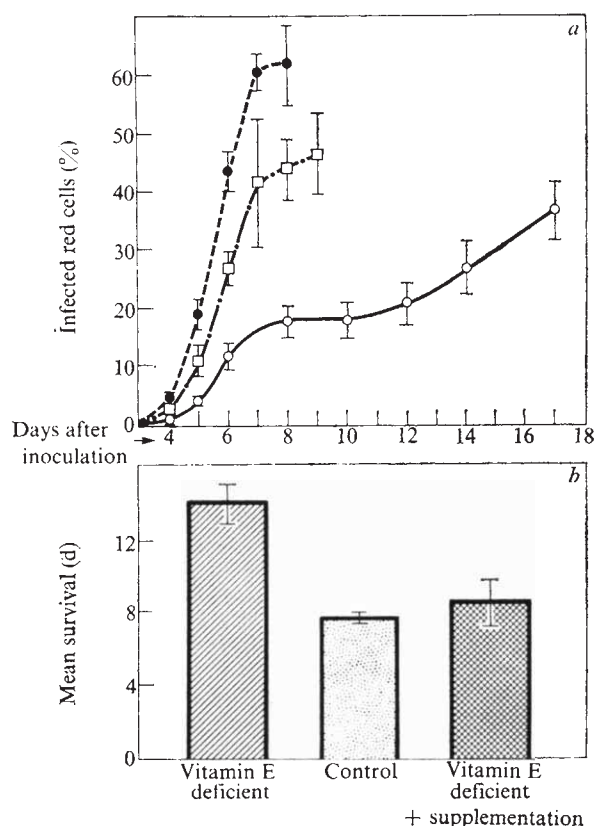


Fig. 1 *a*, Percentage infected red cells in 19 mice fed normal laboratory chow (●--●), 21 mice fed a vitamin E-deficient diet for 60–100 d (○—○), and 8 mice fed a vitamin E-deficient diet for 60–100 d and then resupplemented with vitamin E for 30 d (□--□). Resupplementation of the latter group ended 2 d before inoculation. Each point represents the mean parasite count and the vertical bars through the points represent ± 1 standard error of the mean (s.e.m.). *b*, Mean survival of 19 control, 21 vitamin E deficient and 8 vitamin E-deficient resupplemented mice after inoculation with *P. berghei*. Vertical bars represent ± 1 s.e.m.

sensitivity on the erythrocyte which is similar in many ways to that in G-6-PD deficiency. We now report that vitamin E deficiency moderates the course of *Plasmodium berghei* infection in mice, thus supporting the idea that oxidant sensitivity of host erythrocytes—whether due to G-6-PD or vitamin E deficiency—may limit the severity of malaria.

We recently found that in *P. berghei*-infected mice (1) there is an increase in steady-state methaemoglobin concentration which is directly related to the severity of infection, (2) catalase within infected erythrocytes is extensively inhibited by 3-amino-1,2,4-triazole (an inhibition which requires the presence of H_2O_2) and (3) infected erythrocytes accumulate 10–20 times as much methaemoglobin as do normal red cells when exposed to oxidants generated by ascorbic acid¹. These malaria-infected erythrocytes are thus subject to internally generated oxidants and are also abnormally sensitive to exogenous oxidant stress.

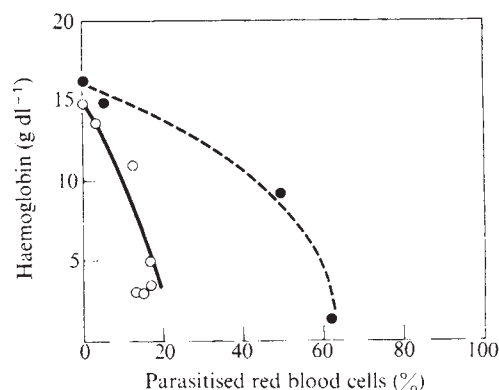
We have now undertaken experiments to determine whether the severity of murine *P. berghei* infection might be diminished by oxidant sensitive host erythrocytes induced by feeding mice a vitamin E-deficient diet. Vitamin E deficiency was used as a model for three main reasons. First, vitamin E normally protects erythrocyte membranes against oxidants and vitamin E deficiency predisposes red cells to peroxide-induced haemolysis^{7–9}. Second, during the earlier experiments we observed that plasma vitamin E concentrations in *P. berghei*-infected mice were approximately half those in uninfected animals, suggesting that oxidants of parasitic origin might increase vitamin E consumption [plasma tocopherol concentrations in 8 uninfected mice =

0.33 ± 0.013 mg per 100 ml and in 14 infected mice 4 d after inoculation = 0.17 ± 0.013 mg per 100 ml; $t=8.03$, $P<0.001$ (Student's *t* test, two-tailed)]. Lastly, recent evidence from some areas of endemic malaria suggested that malnourished African herding peoples, whose diet is normally low in vitamin E, are relatively resistant to severe malaria infection. Feeding these people cereal grains (an excellent source of vitamin E) causes recrudescence of previously undetectable *P. falciparum* infections¹⁰.

Swiss white mice were fed either regular laboratory chow or a vitamin E-deficient diet (Nutritional Biochemicals, Inc.) for 60–100 d. Vitamin E resupplementation of animals previously made E deficient was accomplished by the intraperitoneal injection, every 48 h, of a solution of α -tocopherol (100 mg kg^{-1} body weight) in propylene glycol and absolute ethanol¹¹. In mice on the vitamin E-deficient diet, vitamin E levels reached a nadir after 50 d, as assayed by the peroxide haemolysis test¹² and analysis of plasma tocopherol¹³ [control = 0.34 ± 0.03 mg per 100 ml ($n=16$), vitamin E deficient = <0.10 mg per 100 ml ($n=9$)]. The animals were then inoculated intraperitoneally with 2×10^8 red cells infected with *P. berghei* (NYU-2 strain) as previously described³. Blood was periodically obtained by incision of the caudal veins, and the percentage parasitised erythrocytes was determined by light microscopic examination of a minimum of 500 cells.

Vitamin E-deficient mice are more resistant to *P. berghei* as reflected by slower development of parasitaemia (Fig. 1*a*) and much longer survival (Fig. 1*b*). This resistance seems to be due to an inability of the mature vitamin E-deficient erythrocyte to support the parasite; almost all infected red cells in the deficient mice are reticulocytes whereas in control animals the parasite is found in almost equal frequency in reticulocytes and mature red cells (the ratios of percentage infected reticulocytes to percentage infected mature erythrocytes 7 d after inoculation were: controls, 1:1 and vitamin E deficient, 9:1). Furthermore, deficient mice become more anaemic in proportion to the severity of infection than do normal controls (Fig. 2). The reticulocyte preference of the parasite as well as the severe anaemia in vitamin E-deficient animals suggests that erythrocytes are being infected with normal frequency, but are destroyed abnormally rapidly. It seems that only metabolically competent reticulocytes are able to support full maturation of the parasite. This is consistent with earlier work¹⁴ showing that reticulocytes of vitamin E-deficient rats are resistant to haemolysis induced by exposure to high oxygen pressures *in vivo* and to peroxide *in vitro*. Thirty-day resupplementation of mice still being fed the vitamin E-deficient diet almost fully restores their susceptibility to

Fig. 2 Decline in whole blood haemoglobin concentration of 19 control (●) and 21 vitamin E-deficient (○) mice after infection with *P. berghei*. The points represent the mean haemoglobin and mean percentage infected red cells for all animals.



P. berghei (Fig. 1a and 1b). Furthermore, in these resupplemented animals, the parasite loses its tendency to inhabit only reticulocytes (the ratio of % infected reticulocytes to % infected mature red cells in vitamin E deficient resupplemented mice was 2:1). The diminished susceptibility of vitamin E-deficient mice to malaria infection, therefore, seems to be a specific function of vitamin E-deficiency and not of other deficiencies incurred as a result of feeding the artificial vitamin E-deficient diet.

Quantitative deficiency of G-6-PD is also thought to supply at least a modicum of protection against severe malaria infection. Not only are high frequencies of this enzyme deficiency found in human populations with a history of endemic malaria^{4,5}, but Luzatto has convincingly shown that, in infected heterozygous females, enzyme-normal red cells are much more likely to be parasitised by *P. falciparum* than are enzyme deficient cells⁶. These last results indicate either that merozoites preferentially infect enzyme-normal red cells or that, once infected, the enzyme-normal erythrocytes are much more likely to sustain the parasite for the 48 h requisite for maturation. Our results strongly favour the latter interpretation. Although previous studies of the effect of vitamin E deficiency on the expression of malaria have yielded conflicting results¹⁵⁻¹⁷, we find a markedly slower rate of development of infection in vitamin E-deficient animals. In these mice, the preferential occurrence of parasites within young, oxidant-resistant erythrocytes suggests that this protection is mediated by the premature haemolysis of oxidant sensitive red cells.

In areas where malaria and malnutrition coexist, it may be advisable to administer antimalarial therapy along with dietary supplements.

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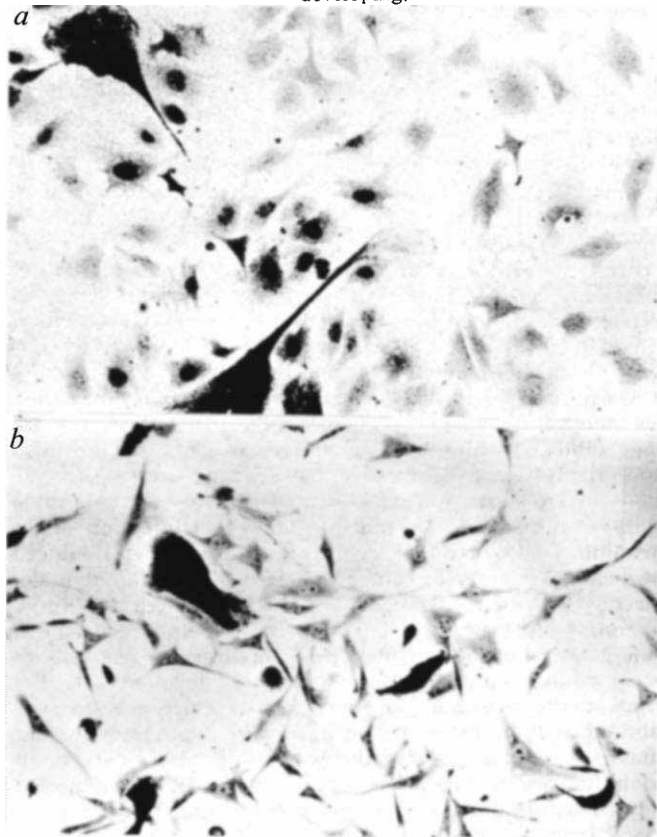
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direct communication can be detected between cultured cells which may be electrically coupled^{1,2}, and which can show metabolic co-operation^{3,4}. Indirect evidence suggests that this direct exchange of ions or small molecules between touching cells, occurs through gap junctions⁵⁻⁷. We have been interested in the question of whether fibroblasts and epithelial cells from the same tissue can interact directly and whether such an interaction is important in the growth of epithelial tumours such as carcinomas of the breast. Although the two cell types are separated by a basement membrane in the normal adult breast, the invasion of the basement membrane, characteristic of malignant cancer cells, allows contact to occur between epithelial cells and fibroblasts. In this situation, the direct communication between the cell compartments of the two types of cell could have a profound effect on the growth and development of the tumour. We show here that normal breast fibroblast and epithelial cells do not directly interact with each other even when they are not physically separated, and speculate that loss of this specificity of communication may be a factor in the development of neoplasia.

Since it has proved difficult to grow the tumour cells from primary human breast carcinomas^{8,9}, we have begun by examining contact-dependent communication between human mammary fibroblasts (HumF) cultivated from explants of terminal ducts, and human mammary epithelial cells (HumE) cultured by the spillage technique from benign tumours^{8,9} or from human milks^{10,11}. Primary culture of both these cell types were thus obtained without enzyme digestion of the tissue. A cell line, MCF-7, derived from a pleural effusion from a patient with breast cancer^{12,13} was also included in the study.

Metabolic cooperation, as it was originally described by Subak-Sharpe, Burke and Pitts^{3,4}, refers to the correction

Fig. 1 a, Transfer of radioactivity from donor lens cell to recipient BHK, b, No transfer of radioactivity from donor HumE cell to recipient BHK. Method as described in Fig. 2 with the modification that cells were exposed to film for 2-4 weeks before developing.



Selective contact-dependent cell communication

INTERACTIONS between animal cells in multicellular organisms are probably important in the coordinated control of growth and function during the normal and abnormal development of specific tissues and organs. Although much of the coordination is lost when cells are separated from each other and grown in tissue culture,

	Donors					
	Lens	RL	BHK	HumF	HumE	MCF-7
Recipients	Lens	+	+	+	+	○
	RL	+	+	○	+	○
	BHK	+	+	+	○	○
	HumF	+	○	+	○	○
	HumE				+	
	MCF-7	○	○	○	○	○

Fig. 2 Primary cultures of HumE and HumF were grown in medium 199 supplemented with hydrocortisone ($5 \mu\text{g ml}^{-1}$), insulin ($10 \mu\text{g ml}^{-1}$) foetal calf serum (15%) and human serum (20%). MCF-7 cells were grown in 199 medium supplemented with calf serum (10%) and insulin ($10 \mu\text{g ml}^{-1}$). Calf lens were grown in 199 with foetal calf serum (10%) and all other cell lines were grown in Dulbecco's modified Eagles medium (DEM) containing 10% foetal calf serum. All cells were grown in Nunclon plastic dishes; those for growth of HumE were coated with collagen which does not affect direct cell interactions between communicating cells (J.T.P., unpublished). Cells were suspended by exposure to trypsin (0.05%) in isotonic Tris buffer (0.02 M) containing EDTA (0.2%). Donor cells were plated in 2 ml of medium at 3×10^3 cells per dish in the medium indicated above, and after overnight growth, medium was removed and $4 \mu\text{Ci}$ of $5\text{-}^3\text{H}$ -uridine (48 Ci mmol^{-1}) were added for 3 h in 2 ml of DEM containing 10% foetal calf serum. The donors were then washed with DEM ($\times 3$) and recipients (2.5×10^5 per dish) were added in fresh DEM+10% foetal calf serum and co-cultured for 3 h in medium without label. The cells were fixed and processed for autoradiography and developed after 7–14 d. Number of grains in donors were between 300 and 2,000. Grains were counted in 150 recipients directly in contact with donors (touching) and in 150 recipients in contact with neither donors nor other recipients (non-touching). HumE cells have been used as donors for all combinations except HumE–HumE because of the limitation in number of cells available. This was considered to be reasonable since all cell combinations tested in a reciprocal manner gave the same result either way.

of a mutant phenotype by co-culture with wild-type cells. Using this method we could follow cell–cell interaction between HumE cells and a mutant fibroblastic cell line TG1 (a strain of polyoma transformed BHK cells lacking hypoxanthine guanine phosphoribosyl transferase). Though TG1 cells are known to communicate with a large variety of fibroblastic cells from several species, our experiments showed that there appeared to be no direct interaction between HumE and touching TG1 cells, suggesting that communicating junctions were not formed between the two cell types. This method is limited, however, to measuring cell interactions which involve mutant fibroblast lines and tests of reciprocity cannot be carried out. Here, we report the application of a method, recently developed by Pitts and Simms¹⁴ for the measurement of direct communication between freshly cultured cells of the same type, and of different types, without the use of mutants.

Donor cells are prelabelled with $5\text{-}^3\text{H}$ -uridine and the transfer of label to the acid-insoluble fraction of unlabelled recipients during subsequent co-culture is followed. Because the $5\text{-}^3\text{H}$ -uridine in the pools of the donor cells is rapidly converted to high molecular weight material¹⁴, the formation of junctions and transfer of radioactive label has to occur within the first few hours of removal of the label in order to be detected. In the experiments reported here, labelled donors have been co-cultured for 3 h with unlabelled recipients as recommended by Pitts and Simms. In addition to the cells from the breast, we have examined a rat liver line (RL) and primary cultures of calf lens (prepared from the explanted lens capsule) as other examples

of epithelial cells and BHK 21 cells as an example of a fibroblastic cell line.

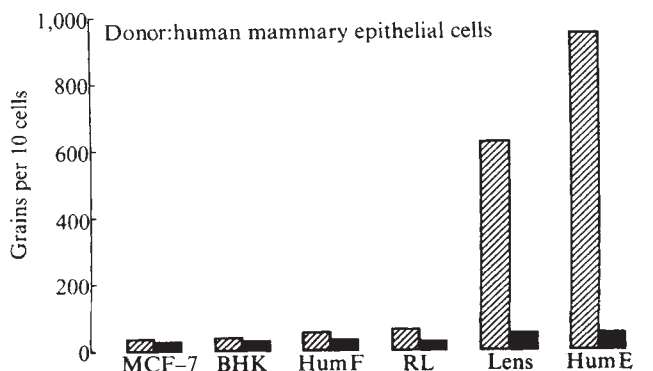
Qualitatively, examples of positive and negative communication can be seen in Fig. 1. To quantitate the transfer of radioactivity, cells were exposed to stripping film for shorter periods of time than that used for the autoradiographs in Fig. 1 so that grains could be counted. For each cell combination, three separate experiments were done using five duplicate plates in each experiment and grains were counted in 10 recipients touching donors, and in 10 recipients touching neither donors nor recipients. Thus the average number of grains in touching and non-touching recipients is calculated in each case from 150 values. The results are presented in Figs 3 and 4 and summarised in Fig. 2.

From Figs 2 and 3 it can be seen that while HumE cells communicate directly with other HumE cells (or lens cells) and HumF with HumF (or lens) no such direct communication could be detected between HumE and HumF. Previous work following direct interactions between cell lines has indicated that cells which can form communicating junctions do so without specificity as to cell type¹⁵. The results reported here demonstrate, under the conditions of the experiment, a specificity in the ability of cells to communicate directly with other cell types, and we can refer to HumE and HumF as “selective communicators”. The RL cells are also “selective communicators” (Fig. 4) in that, while communicating through contact with other RL (or lens cells), they do not under the same conditions donate ^3H -uridine to HumF. However, when a fibroblastic cell line, BHK, is used as recipient instead of primary cultures of HumF, some specificity is lost and a certain amount of radioactivity is transferred from the RL donor to the touching BHK recipients. The HumE cells, however, do not donate radioactive label during 3 h of co-cultivation even to fibroblastic cell lines.

A few but not all RL cells co-cultured with and touching HumE donors showed an increased grain count over non-touching RL. In Fig. 4 this result shows up as a small increase in the average number of grain counts in touching over non-touching cells, and is represented as positive and negative in Fig. 2. The result suggests that communicating junctions can be formed between the two types of epithelial cells but not with the ease and rapidity with which they are formed between epithelial cells of the same type.

Calf lens cells are unique among the cells we have examined in that they donate radioactivity when prelabelled with $5\text{-}^3\text{H}$ -uridine to all the cells tested, (except MCF-7—see below) that is, they are “non-selective communicators”. Thus there is not a general failure of all cells of epithelial origin to communicate directly with fibroblasts. The readiness

Fig. 3 Selective communication of HumE cells with other cell types. For method see legend to Fig. 2. Hatched bars, grains in touching recipients; black bars, grains in non-touching recipients.



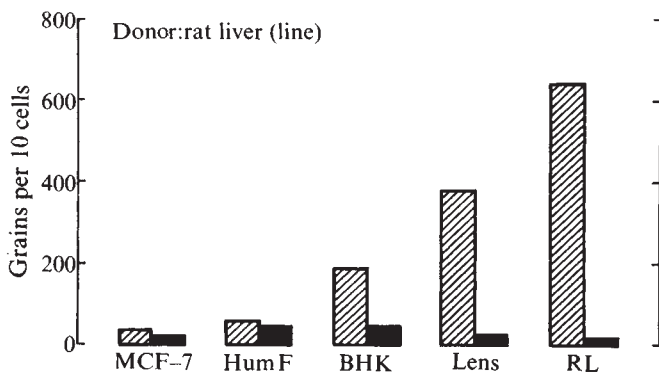


Fig. 4 Selective communication of RL cells with other cell types. Hatched bars, grains in touching recipients; black bars, grains in non-touching recipients.

with which the lens cells form communicating junctions may well be related to the abundance of gap junction proteins which are found in their membranes¹⁶.

Metabolic cooperation studies as well as electrical resistance measurements have shown that L cells and some tumour cells and cell lines do not form communicating junctions either with themselves or any other cells^{17,18}. MCF-7 cells resemble L cells in that they seem to be unable to communicate directly either with themselves or with any other cells tested.

The data reported here demonstrate that the intracellular pools of uridine or its nucleotide¹⁴ in HumE cells do not readily communicate directly with the corresponding intracellular pools in HumF cells under conditions where there is exchange between the pools of cells of the same type. This suggests an important possibility, namely, that in certain mixed cultures such as HumE and HumF, two cellular compartments may exist which do not interact by transfer of molecules. Indeed in mixed cultures of these two cell types, the cells grow physically separated from each other. If the results can be translated to the *in vivo* situation, they suggest that even if the basement membrane did not physically separate the two types of cell, they might form junctions only with cells of the same type. Such a specificity governing interactions between different cell types could be an important factor in the normal regulation of growth in multicellular organisms and further studies on interactions between cells in other tissues and organs are indicated.

Our results have been obtained with cells obtained from normal or hyperplastic breast and as yet we do not know whether the primary carcinoma cells behave like the normal epithelium. It has been suggested by Loewenstein¹⁹ that the complete loss of the ability to directly communicate with other cells, which is observed in some tumour cells (including the MCF-7 line used here) could account for the lack of response in these cells to normal growth control signals which may come through direct cell contact. The existence of specificity in direct cell-cell communication, as demonstrated here, opens up the possibility, which can be experimentally tested, that a loss of specificity in cell-cell interactions could be associated with abnormal cell growth.

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Specificity of junctional communication between animal cells

MANY types of animal cells, in culture and *in vivo*, form intercellular junctions which are freely permeable to small cellular ions and molecules but not to macromolecules¹⁻¹⁰. These junctions are probably the gap junctions seen by electron microscopy¹¹. Cells coupled by such junctions share their metabolites and small control molecules and respond jointly to changing demands on metabolism. Intercellular control of enzymic activity^{12,13} and metabolic interdependence causing controlled cell proliferation⁷ have both been observed in mixed cell populations in tissue culture. These integrative effects of junctional communication make a tissue behave as a homogenous unit rather than a collection of different cells.

The ubiquitous occurrence of gap junctions *in vivo* suggests that junctional integration is a widespread phenomenon, but one can conceive of instances where integration is a disadvantage and independence is important. Such situations would require specificity of junctional communication to functionally isolate different cell types either within the same tissue or in different contiguous tissues.

Examination of cultured vertebrate cells for their ability to form intercellular junctions has, until now, revealed only two classes. Cell types in one class (which includes about 90% of the cell types so far examined) form junctions with all other members of the class and with no detectable tissue or species specificity (ref. 9 and B. Kukulska, P. Ferry and J. D. P., unpublished). Cell types in the other class appear to be genetically incapable of junction formation and so never form junctions either among themselves or with any other cell type^{7,14,15}. However, in this paper we describe a tissue culture system which is different from these others in that it shows a form of specificity of junction formation.

Rat liver cells (BRL cells¹⁶) formed junctions rapidly and extensively with each other. Hamster fibroblasts (BHK21/13 or C13 cells¹⁷) similarly formed junctions very efficiently among themselves⁷. But in mixed cultures junctions were not formed or formed only very slowly, between BRL and BHK cells.

Uridine nucleotide exchange can be used as an assay of intercellular junction formation. Pitts and Simms^{9,10} have shown that uridine nucleotides, but not RNA, are freely transferred between cells joined by gap junctions. However, because nucleotides cannot cross the normal cytoplasmic membrane, they are not transferred to cells which are not in contact or to cells which are unable to form gap junctions.

Donor cells were prelabelled for 3 h with 5-³H-uridine and washed with unlabelled medium. Such donor cells contain 5-³H-uridine nucleotides and ³H-RNA but no detectable 5-³H-uridine. Unlabelled recipient cells were added to the washed donor cells and the mixtures co-cultured. After

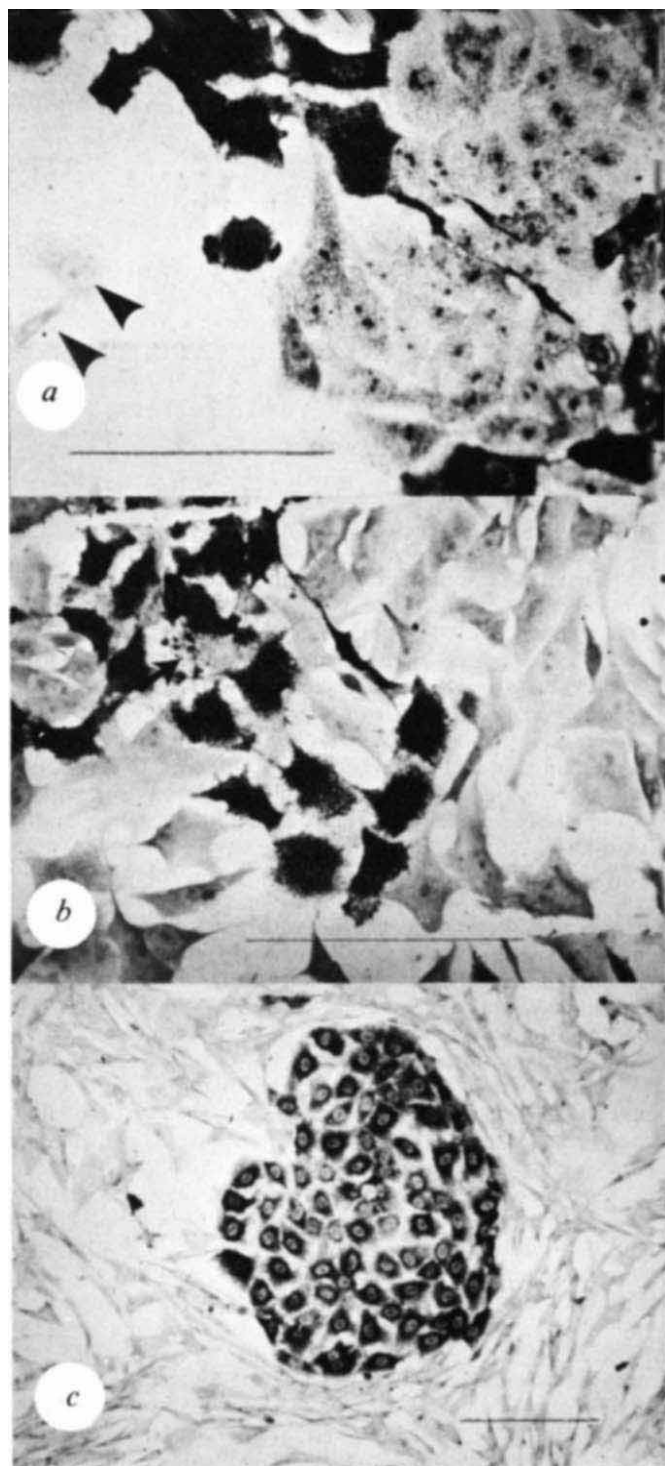


Fig. 1 Donor cells (5×10^4) in 5 ml Eagles medium (Glasgow modification, supplemented with 10% calf serum) were grown overnight at 37°C in 5 cm plastic dishes containing 13 mm diameter sterile coverslips. The cells were labelled for 3 h with $5\text{-}^3\text{H}$ -uridine ($5 \mu\text{Ci ml}^{-1}$; 27 Ci mmol^{-1}), then washed four times with unlabelled medium. Unlabelled recipient cells (5×10^5) were added to the prelabelled washed donor cells and the mixtures co-cultured for 3 h (a,b) or 18 h (c). The cultures were fixed with formal-saline. The coverslips were washed for 5 min at 0°C twice in 5% TCA and twice in water and then rinsed in ethanol. The dried coverslips were mounted on microscope slides and processed for autoradiography (Ilford L4 emulsion). The autoradiographs were stained with Giemsa. Heavily labelled cells are donor cells and unlabelled or lightly labelled cells are the added recipients. a, BRL donors to BRL recipients (note two unlabelled recipient cells not in contact with donor cells, arrowed); b, BRL to C13 (note one labelled recipient, arrowed); c, BRL to C13 (showing island of epithelial BRL cells). Scale bars: 50 μm .

co-culture, the cells were fixed, acid washed and processed for autoradiography. Light-labelling over recipient cells due to uridine nucleotide transfer from the heavily labelled donor cells indicates junction formation. For details of method see description of Fig. 1 and ref. 10.

Junctions were formed between all BRL cells in contact (Fig. 1a) and between all C13 cells in contact. However, under the same conditions, junction formation between BRL and C13 cells was very rare even though they appeared by light microscopy to be close enough to make contact.

The extent of nucleotide transfer in the co-cultures was quantitated by counting grains over recipient cells. Primary recipients (recipient cells in direct contact with labelled donor cells) were then classified as labelled or unlabelled. Secondary recipients (recipient cells in direct contact with labelled primary recipients) were similarly classified.

The percentages of primary recipient cells labelled (the extent of transfer) in both homologous and both heterologous donor-recipient mixtures are given in Table 1. No instance was found of an unlabelled recipient BRL cell in contact with donor BRL and only two of 328 recipient C13 cells in contact with donor C13 cells were unlabelled. This very extensive junction formation is similar to that found with a wide variety of cell types^{8,7,10}.

A new situation arose, however, when junctional communication was examined in the heterologous mixtures. Even though there was close apposition between donor cells and potential recipients, detectable transfer occurred between less than 6% of the cell pairs examined after 3 h co-culture (Table 1). In the few cases where there was transfer (5.4% for BRL to C13 and 4.9% for C13 to BRL) the amount of transfer was less than that between cells of the same type, but not greatly so (Table 2 and compare the number of grains over the one labelled recipient in Fig. 1b with those over the labelled recipients in Fig. 1a). In the heterologous cultures, homologous transfer always occurred from every labelled primary recipient cell (Table 1).

The extent of interaction in the heterologous mixtures appears to be a function of the time of co-culture. If the donor and recipient cells were co-cultured for only 1 h the extent of interaction was less than 2% (1.9% for BRL to C13, 1.2% for C13 to BRL), but after 18 h it increased to almost 50% (44% for BRL to C13, 41% for C13 to BRL). In the homologous mixtures the extent of interaction was 100% at both 1 h and 18 h.

It seems that BRL cells do not form junctions readily with C13 cells but, in at least some cases, they do after

Table 1 Specificity of junction formation

Donor cells	% Recipient cells labelled			
	BRL cells		C13 cells	
	Primary recipients	Secondary recipients	Primary recipients	Secondary recipients
BRL	100	100	5.4	100
C13	4.9	100	99.4	100

Donor cells (2×10^4) were labelled for 3 h with $5\text{-}^3\text{H}$ -uridine ($0.2 \mu\text{Ci ml}^{-1}$) and then washed four times with unlabelled medium (see Fig. 1). Unlabelled recipient cells (8×10^5) were added and the mixtures co-cultured for 3 h. After fixing, washing and processing for autoradiography, grains were counted over labelled recipient cells in direct contact with donor cells. The heavily labelled donor cells are readily distinguished from the lightly labelled recipients. Primary recipients are recipients in direct contact with a donor cell. Secondary recipients are recipients in direct contact with a primary recipient cell. Recipient cells with < 5 grains per cell were classified as unlabelled, with > 30 grains per cell as labelled. The background count was < 4 grains per cell. Cells with grain counts between 6 and 29 were ignored (7 out of 1,264 counted). More than 200 donor-recipient pairs were examined in each mixture (except for the secondary recipients in the heterologous mixtures where insufficient examples could be found and only 70 primary recipient-secondary recipient cell pairs were counted).

prolonged contact. Once junctions have been established, the rate of nucleotide transfer is similar to that between cells of the same type. The grain counts over primary recipients are lower when the cell types are different (Table 2) but if the time course of junction formation in these heterologous co-cultures is taken into account, then the average grain counts (average for cells which formed junctions early and cells which formed junctions later in the 3 h period) suggest similar rates of transfer.

Table 2 Extent of nucleotide transfer

Grain counts over primary recipient cells	
BRL to BRL	63±19
BRL to C13	48±20
C13 to C13	88±29
C13 to BRL	53±16

For experimental conditions see Table 1. The figures are mean values of grain counts over either 200 (BRL to BRL and C13 to C13) or 70 (BRL to C13 and C13 to BRL) primary recipient cells with standard deviations.

Junctional communication between BRL and C13 cells, once established, appears to be similar to that in other systems and it is possible that the junctions are identical. It may be, therefore, that the frequency of cell-cell contact and not a difference in junctional subunits underlies the specificity of junction formation. The frequency with which the membranes of homologous cell pairs come close enough together to allow junction formation may be much higher than that possible for BRL-C13 pairs.

In cell systems where junctions form quickly and extensively, communication is maintained (even through cell division) until the cells are physically separated (for example by trypsinisation or mechanical means). However, in the heterologous cell systems described here, lightly labelled cells (recipients) not in contact with a more heavily labelled cell can be found occasionally (0.8% of labelled recipients after 18 h co-culture). This ability to break communication, even at this very low rate, is strikingly different from the homologous systems where all labelled recipient cells were in contact with heavily labelled cells. This may reflect the relative weakness of the heterologous cell-cell contact.

In both mixed and unmixed cultures the epithelial-like BRL cells tend to form islands or domains and thus maintain maximum cell-cell contact. In the mixed cultures these islands are not invaded by the fibroblasts which suggests that the BHK cells cannot satisfy the BRL cells' requirement for intercellular contact. This inability to form adequate contact between the two cell types may explain both the culture morphology and the specificity of junction formation.

This paper describes a type of specificity in the formation of junctions between cells in tissue culture. The accompanying paper¹⁸, using cells in primary culture, with similar results, suggests the specificity we have analysed may be a general phenomenon.

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Response to epidermal growth factors of cultured human mammary epithelial cells from benign tumours

IN a pilot study on the culture of unselected human breast tumours it was found that fibroadenomas which were disrupted mechanically (without enzyme treatment) consistently gave rise to epithelial cell cultures uncontaminated by fibroblasts¹. These epithelial cells, which were termed E cells, could not be distinguished from the major class of epithelial cells isolated from lacteal secretions of healthy women^{2,3}. It is not known whether the fibroadenoma yields epithelium which is truly normal (and is thus a fibroma), or whether the epithelium is abnormal in a way not yet identified. Nevertheless, the reproducibility of the cultures from these tumours, together with ease of storage at low temperature, makes them suitable for detailed studies on mammary epithelial growth, and for comparison, not only with lacteal secretion cells, but with the more heterogeneous cultures from carcinomas. We describe here some of the growth characteristics and requirements of epithelial cells from fibroadenomas, and in particular their response to epidermal growth factor⁴, but lack of response to more familiar mammotrophic hormones.

Cultures were made from stored frozen aliquots of uncultured cell suspensions from mechanically disrupted fibroadenomas removed in the ICRF Breast Unit at Guy's Hospital. The suspensions, which consisted predominantly of clusters of 10-100 cells were distributed in growth medium (see legend to Fig. 1) to give 100-500 clusters per 30 mm dish as required (see legend Fig. 1). Experiments were repeated with fresh cultures from the same or different tumours. Except for cell yield, there was little variation among fibroadenomas, nor among replicate or successive cultures from the same tumour. Viability was unaffected by storage in liquid nitrogen for up to 8 months.

When incubated at 37 °C, 5-15% of the cell clusters attached and spread to give characteristic smooth-edged islands of tightly adjoining cells typical of the E cells known to exhibit epithelial junction systems and microvilli. Islands of fibroblast like cells were found in cultures from only two out of 50 tumours, and no mixed (epithelial-fibroblastic) islands were seen. Though the epithelial islands were uniform in appearance however, we have no evidence that they are all identical or individually homogenous.

The islands enlarged during the following 3 weeks with approximately a 2-d average doubling time to reach a maximum of about 10⁵ cells. After 3 weeks in culture, however, growth consistently came to a halt, as shown by lack of further enlargement of islands, and absence of DNA synthesising cells detected by autoradiography after 48 h exposure to ³H-thymidine. A similarly limited growth period has been found for E cells from lacteal secretions⁵. Subculture of the growing cells by suspension in 0.025% trypsin gave similar islands (plating efficiency 2% and 7% in two experiments) but did not extend the total growth period.

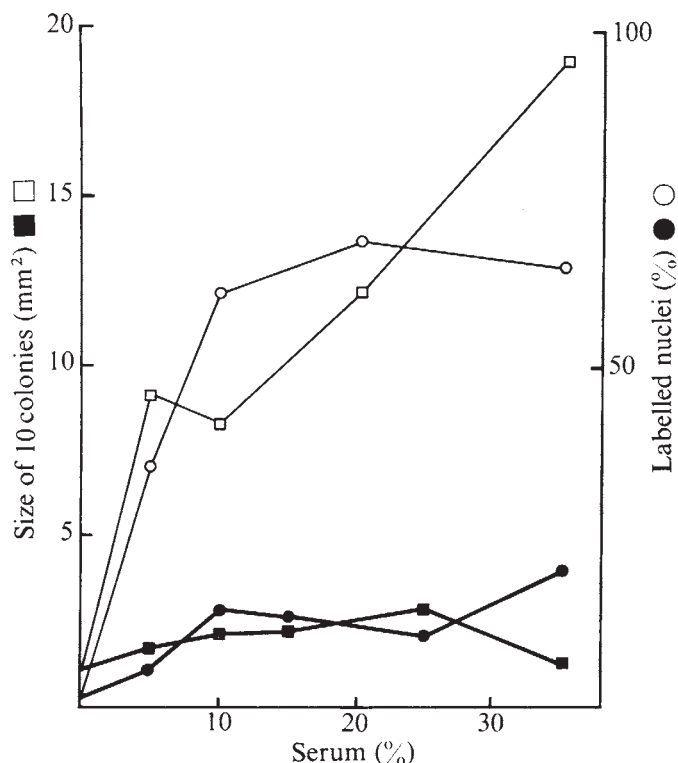


Fig. 1 Response of mammary epithelial cells to human serum and foetal calf serum. Fibroadenomas were disrupted mechanically and the resulting suspension filtered through gauze and allowed to sediment on the bench for 30 min. The loose deposit was suspended in medium with 10% dimethyl sulphoxide to give 10^3 to 10^4 cell clusters per ml and stored in 1 ml aliquots at -160°C . For culture, samples were thawed and distributed in 30 mm dishes to give several hundred clusters per dish in 2 ml volumes of the following medium: medium 199 with Earle's salts, buffered with sodium bicarbonate (3.7 mg ml^{-1}) and containing L-glutamine ($100\text{ }\mu\text{g ml}^{-1}$), glucose (1 mg ml^{-1}), streptomycin ($100\text{ }\mu\text{g ml}^{-1}$), penicillin (100 U ml^{-1}), supplemented with hydrocortisone ($5\text{ }\mu\text{g ml}^{-1}$), insulin ($10\text{ }\mu\text{g ml}^{-1}$) unactivated filtered ($0.22\text{ }\mu\text{m}$ millipore) foetal calf serum (15%) and pooled human serum (20%). Medium was changed twice weekly. After 14 d single cultures were washed twice in serum-free medium, changed to 1 ml of medium 199 (with hydrocortisone and insulin as above) and to either human serum (open symbols) or foetal calf serum (closed symbols) in the concentrations shown. After a further 2 d ^3H -thymidine ($2\text{ }\mu\text{Ci ml}^{-1}$, 20 Ci mmol^{-1}) was added for 48 h. The labelling index (%) was determined by autoradiography and based on counts of 800 to 2,000 cells distributed in 10 randomly distributed islands per plate. Colony size was estimated from the total area of the same 10 colonies. Cell density was not affected by island size.

The studies reported here were carried out between days 9 and 18 of primary culture.

In our earlier study the standard growth medium contained 15% foetal calf serum, but an additional 20% human serum was added when it was observed that epithelial growth was enhanced. To investigate further the serum requirements, growing cultures of E cells were washed in serum-free medium and replaced in medium without serum, or with varying quantities of either human or foetal calf serum. As judged by labelling index, or cell numbers derived from island size (Fig. 1) E cell growth is maintained in human serum, proportionately to dose, but there was little response to calf serum. Various batches of both sera were tested with the same results. Mammary fibroblasts, on the other hand, obtained from enzyme-digested fibroadenomas, or in rare instances from mechanically disrupted tumours, responded equally well to foetal calf serum and human serum (data not shown). Since the response of epithelium to human serum was not inhibited in mixtures with calf serum, we assume that the latter is defective in essential growth factors for epithelium. The requirement is

not species specific however because adult swine serum (but not adult cow serum) will also support human mammary cell growth.

The activity of human serum was considerably reduced by absorption for 1 h with activated charcoal (Norite 5×2, 10 mg ml^{-1} ; Harrington, London). In a preliminary attempt to identify the active constituents, certain hormones were added to washed cultures of E cells in place of serum. Oestradiol (1 and 10 ng ml^{-1}), human and ovine prolactin (0.1 and $1\text{ }\mu\text{g ml}^{-1}$), insulin ($10\text{ }\mu\text{g ml}^{-1}$), and hydrocortisone ($5\text{ }\mu\text{g ml}^{-1}$) were unable to maintain DNA synthesis when used alone, or in various combinations, in cultures from several different fibroadenomas. Mouse epidermal growth factor (EGF), however, consistently maintained DNA synthesis in cultures from all fibroadenomas tested. Figure 2 shows that 1 ng ml^{-1} ($1.7\times 10^{-10}\text{ M}$) was effective and the response increased up to 10 ng ml^{-1} . At higher concentrations the response was not increased proportionately (and sometimes fell) so 10 ng ml^{-1} was routinely used. When 0.1% serum was added (insufficient to maintain DNA synthesis alone) there was a small enhancement at the lower EGF concentrations.

Table 1 gives data from three experiments and shows that EGF increases the proportion of DNA synthesising cells, as well as overall synthesis measured by ^{14}C -thymidine incorporation. It will be seen that the response to EGF is unaffected by hydrocortisone, with or without insulin, but is slightly enhanced by 0.1% serum as already shown. Other experiments have shown that insulin alone, oestradiol, and prolactin (concentrations as above) or bovine serum albumin ($2.5\text{ }\mu\text{g ml}^{-1}$) have no effect on response to EGF. EGF is known to stimulate mouse and human fibroblasts, so the substantial response of mammary fibroblasts, also shown in Table 1, is not surprising.

Thus far we have only shown that human serum and

Fig. 2 Response of human mammary epithelial cells to epidermal growth factor (EGF). Fibroadenoma cultures as in Fig. 1. After 12 d in growth medium cultures were washed twice in serum-free medium and EGF was added in doses shown to pairs of dishes in 1 ml volumes of medium 199 (with hydrocortisone and insulin) either with (squares) or without (circles) 0.1% human serum. After 3 d medium was changed, with same additions, and ^{14}C -thymidine ($0.05\text{ }\mu\text{Ci ml}^{-1}$, 50 mCi mmol^{-1}) was added for a further 2 d before acid extraction and scintillation counting, shown separately for each culture.

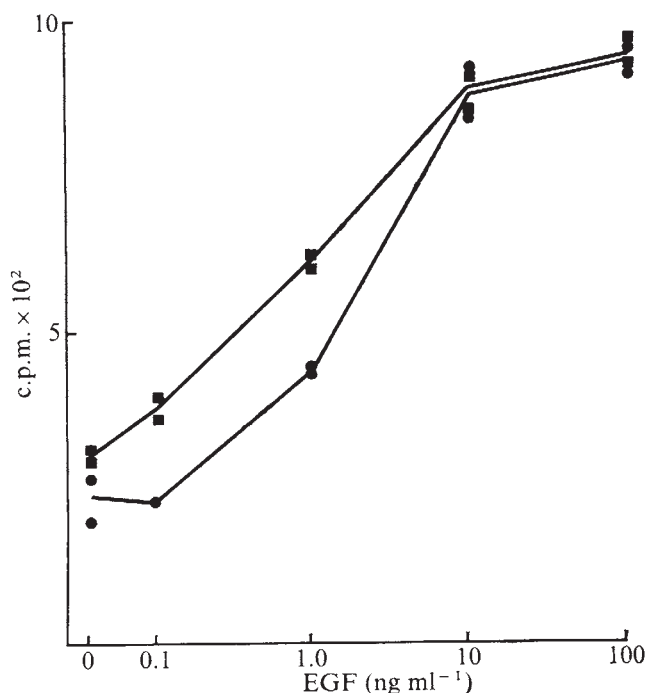


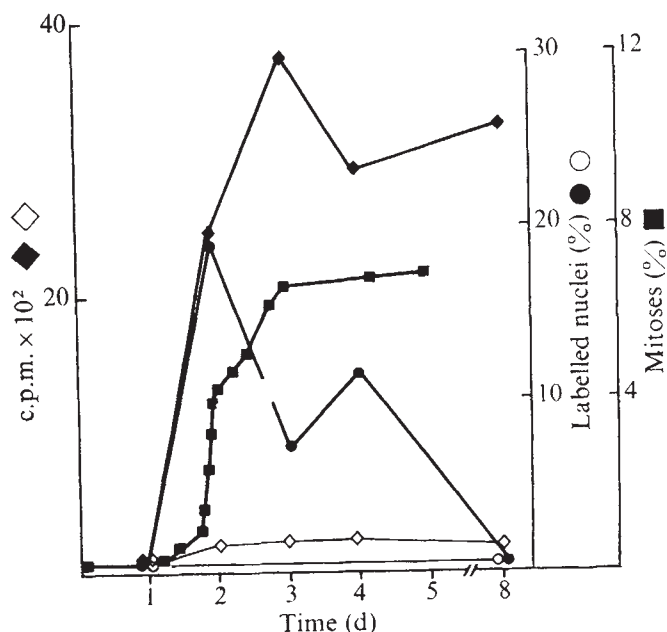
Table 1 Effect of epidermal growth factor, hydrocortisone, and insulin on thymidine incorporation by human mammary epithelial cells and fibroblasts

Additions		Experiment 1 (% labelled)	Experiment 2 (c.p.m.)	Experiment 3 (c.p.m.)		Experiment 3 (c.p.m.)	
		Epithelium FA T99 Growing	Epithelium FA T286 Growing (a)	Epithelium FA T232 Growing	Epithelium FA T232 Quiescent	Fibroblast MP N2F Growing	Fibroblast MP N2F Quiescent
nil		0.8	17; 22	40; 38	—	—	—
Hydrocortisone	—	0.1	26; 44	124; 28	47; 50	75; 59	169; 129
Insulin	—	17.7	453; 486	778; 597	—	—	225; 130
EGF	—	14.0	388; 411	629; 388	321; 297	208; 235	2,544; 2,400
Hydrocortisone	—	—	13; 48	26; 33	—	—	3,205; 2,389
Insulin	—	—	440; 452	832; 597	—	—	—
EGF	—	—	4,303; 4,483	—	4,680; 5,704	690; 754	29,920; 30,499
Human serum	—	35.6	—	—	—	—	16 795; 16,903

Epithelial cultures as in Fig. 1 from three separate fibroadenomas: fibroblast cultures by plating dispersed cells after collagenase and hyaluronidase digestion of normal mammary tissue removed at mammoplasty (10^3 cells per 30 mm dish). After 9 d in growth medium the cultures were washed twice with serum-free medium and treated as follows: for growing cultures 1 ml medium 199 was added, with the additions shown, for 3 d. The medium with the same additions was then replaced for a further 2 d with ^3H -thymidine or ^{14}C -thymidine. In experiment 2a 0.1% human serum was present throughout. For quiescent cultures medium 199 (with hydrocortisone and insulin) was added for 3 d to all cultures, then replaced for 2 d with the additions shown and ^{14}C -thymidine. The labelling index (experiment 1) was estimated by autoradiography and counts on all labelled nuclei in 10 randomly selected colonies, comprising at least 3,800 cells. Incorporation of ^{14}C -thymidine (experiments 2 and 3) in duplicate cultures, shown as counts per minute per culture above background (10 min counts, background exp. 2: 16 c.p.m., exp. 3: 29 c.p.m.). Cell numbers were not high enough for estimation of DNA. Concentrations of additions were as follows: hydrocortisone $5 \mu\text{g ml}^{-1}$, insulin $10 \mu\text{g ml}^{-1}$, mouse epidermal growth factor 10 ng ml^{-1} (25 ng ml^{-1} in exp. 1), unactivated pooled adult human serum 20% (35% in exp. 1).

EGF can maintain DNA synthesis in already growing cultures of mammary epithelial cells. In medium 199 alone, with or without insulin and hydrocortisone, such cells stop synthesising DNA and dividing, and remain quiescent with a labelling index (48 h exposure to ^3H -thymidine) of less

Fig. 3 Time course after restimulation of quiescent mammary epithelial cells with epidermal growth factor (EGF). Fibroblast cultures as in Fig. 1. After 10 d in growth medium, cultures were washed twice in serum-free medium, and left in 1 ml of medium 199 (with hydrocortisone and insulin) for 3 d. The medium was then changed and EGF (10 ng ml^{-1}) added to about half the cultures. The remainder were controls without EGF. At this stage, one set of cultures, with and without EGF, also received ^{14}C -thymidine, and thereafter one culture was removed each day for acid extraction and scintillation counting, shown as cumulative incorporation per culture. The other set, with and without EGF, was exposed to ^3H -thymidine added on successive days for 24 h periods, before autoradiography and estimation of labelling index (based on counts of at least 3,000 cells distributed in six randomly selected islands). Closed symbols: with EGF; open symbols: without EGF. Mitoses were determined by time lapse cinematography on one culture with EGF and are shown as the cumulative number in a single confluent area within one island and containing about 400 cells.



than 1% for several weeks without morphological evidence of cell damage. Table 1, experiment 3, shows that both serum and EGF can reinitiate DNA synthesis in such quiescent cultures of E cells (and also mammary fibroblasts), while in Fig. 3, a time course from another experiment, shows that reinitiation of DNA synthesis occurs 24 to 48 h after addition of EGF and is accompanied by mitosis, but is limited to one cycle. While human serum or EGF invariably maintain DNA synthesis in growing culture, however, the response of quiescent cultures to restimulation was variable and in the case of EGF sometimes undetectable. Lack of reinitiation was not a feature of particular tumours, or time in culture, and is at present unexplained.

EGF which is a stable and highly purified polypeptide hormone isolated by Cohen and his colleagues⁹ is mitogenic for murine⁶ and human fibroblasts^{7,8} for mouse mammary epithelial cells in organ culture⁹ and rat mammary epithelium in cell culture (P. S. Rudland, R. D. Hallows, H. Durbin, and D. Lewis, unpublished). A polypeptide with similar biological properties has recently been isolated from human urine¹⁰. In these systems serum or albumen is also required for EGF activity, whereas in the cultures described here EGF invariably maintained, and, less regularly, reinitiated, DNA synthesis in defined medium in the absence of added protein. Admittedly, serum might have been carried over despite repeated washing of the cultures, but is unlikely to have been an important factor since re-addition of serum had little effect on the EGF response. Though EGF could not replace the maximum effect of human serum (see Table 1), thus implying the need for other factors, it was effective at a physiological level, such as reported in mouse plasma¹¹.

The lack of response of our cultures to known mammotropic hormones, such as prolactin and oestradiol, may be due to selection of an unresponsive cell, or inadequate culture conditions, perhaps even the lack of a required interacting heterologous cell. It is clear, however, that other less familiar hormones such as epidermal growth factor should also be investigated for their role in the physiology and pathology of the mammary gland.

We would stress that this report concerns mammary epithelium studied within 3 weeks, or about 10 average cell doublings, of the origin *in vivo*, and in the absence of fibroblasts. In addition to the failure to respond to foetal calf serum, which may have nothing to do with EGF, the

epithelial cells differed from human fibroblasts in their much shorter life span. This characteristic of freshly cultured epithelial cells may result from inadequate culture conditions, but alternatively it may be a feature of cells which are shed after a limited proliferative phase.

Finally, since the cells will grow alone in the absence of fibroblasts, we can conclude that interaction with stromal cells is not an absolute requirement for growth of epithelium. This does not mean that interactions with non-epithelial cells are excluded, and indeed other investigations from our laboratory (ref. 3 and unpublished data) show that fibroblasts from the breast and other sources, as well as macrophages all affect the growth of mammary epithelium. But the starting point for such studies must be a source of freshly derived epithelium, free of heterologous cells, as described here.

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Cell-cycle initiation in yeast follows first-order kinetics

THE mitotic cell cycle is an orderly sequence of events, leading to cell division. In mammalian cell lines, a single event in the cycle exhibits first-order kinetics¹, apparently dividing a cell's lifespan into a probabilistic state and a deterministic phase, termed A and B respectively. This rate-limiting event occurs during the G₁ phase^{2,3}, but its relation to the resting state and to the initiation of the cell cycle is unclear. In this paper we suggest that a probabilistic event exists in the mitotic cell cycle of yeast and that it

can be identified with the initiation of a new cycle from the resting state.

The yeast *Saccharomyces cerevisiae* signals a new cell cycle by initiating a bud. The size of the bud, as it develops, is correlated with the phase of the cell in the cycle. Temperature-sensitive cell-division cycle (*cdc*) mutants were isolated, which cannot proceed beyond a particular phase of the cycle at the restrictive temperature^{4,5}. In addition to the mitotic cell cycle which is common to both haploid and diploid strains, two other developmental pathways are observed in yeast. One pathway, conjugation, is normally an alternative to the cell cycle in haploid cells, while meiosis, the other pathway, is an alternative only in diploid cells. The three pathways intersect at a common point, termed "start", at which emergence to any one of them occurs⁶. The environmental conditions determine which developmental pathway is chosen by the cell when it is at "start".

Yeast cells may be arrested before "start" as single, unbudded cells by one of three methods^{5,6}, namely by starvation into stationary phase, by incubation at the restrictive temperature of a *cdc* mutant that arrests at this point of the cell cycle and by exposure of haploid cells to the mating hormone of the opposite mating type. We have used the two latter methods to arrest two exponentially growing populations of a haploid A strain that carries the mutation *cdc25* (ref. 7), which is a "start" mutation, and followed bud emergence after release of the blocks (Fig. 1). In both populations buds emerged asynchronously following similar first-order kinetics with a defined lag period of about 45 min (see also straight-line curve semi-logarithmic plotting of data in insert of Fig. 1). Similar results were obtained with two other "start" mutations, *cdc28* and *cdc35*. For comparison, data are given of the release of the temperature block of another mutant, *cdc24*, which also arrests prior to bud emergence but at a later stage than "start" (Fig. 1). In the latter case buds appear with a much better synchrony than after

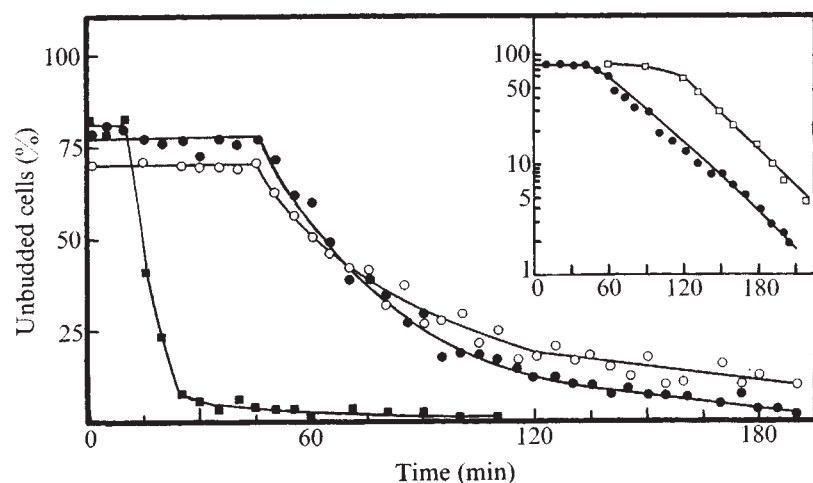


Fig. 1 Kinetics of bud initiation following release from blocks at "start" and at a later stage. The haploid strain 352/1 was derived from Hartwell's strain BR-205-2A. It is of mating type a and contains a temperature sensitive "start" mutation *cdc25* and the markers *ade2* and *ura1*. The haploid 337, named by Hartwell 182-6-3A is of mating type a and contains the mutation *cdc24* and the markers *ade1*, *ade2*, *ura1*, *lys7*, *his7*, *try1*, *gal1*. The cells were grown in YEPD medium, containing 10 g yeast extract, 20 g bacto peptone, and 20 g dextrose in 1 l of distilled water. Exponentially growing cells were blocked before "start" by a 3.5-h incubation at the restrictive temperature 36 °C in a shaking bath, or by a 2.5 h incubation at 25 °C in a medium where α cells were previously grown to high density and removed from the medium by centrifugation. The temperature block was released by transferring the culture to 25 °C and the α -factor block was removed by centrifugation and resuspension in fresh YEPD. In order to separate cell aggregates, the cultures were sonicated upon release from the blocks with the microtip of sonifier B-12 (Branson) for two 5 s periods (power 125 W). After sonication the cells were diluted to 2×10^6 cells ml⁻¹ and plated on YEPD solid medium (2% agar) at 25 °C. Samples were scored at 5 min intervals. The percentage of single unbudded cells was determined by counting at least 300 cells for each sample under the microscope. (●) strain 352/1 (*cdc25*) following release of temperature block. (○) strain 352/1 following release of α -factor block. (■) strain 337 (*cdc24*) following release of temperature block. The insert shows a semi-logarithmic plotting of 352/1 following release of temperature block (●) and the appearance of the second buds in *cdc24* (□). Note: similar kinetics were obtained with 352/1 after a 6-h block at 36 °C.

the former two blocks, following a well timed course of events which is initiated uniformly in all the cells that are released from the block. The synchronous budding of *cdc24* suggests that asynchrony is not a general feature of budding or of the *cdc* mutants, but is rather a consequence of the existence of a rate-limiting step in the cycle. After passing this step, the appearance of the second buds in *cdc24* is asynchronous and follows similar kinetics to the appearance of the first buds in *cdc25* (insert of Fig. 1).

When does the rate-limiting step occur? It must be located before the *cdc24* block because release of the latter allows for synchronous pursuance of the cell cycle. With respect to the "start" point, which is defined by the *cdc25* block, the rate-limiting step could occur either after, or at this very point. However, it is difficult to monitor the "start" event because it occurs up to 45 min before bud emergence. In order to distinguish between the two alternatives, the following experiment was conducted in which the actual rate of "start" emergence was monitored. *cdc25* cells were released after being blocked at the restrictive temperature. Following different periods at the permissive temperature, samples were returned to the restrictive one. The experiment was stopped when the fraction of budded cells at the permissive temperature reached a sufficiently high value (80% budding). In each sample the frequency of buds at the time of return was compared to the frequency recorded at the end of the experiment.

One of two sets of results can be expected according to the two alternatives. The first alternative is that the rate-limiting step is located after "start" and release from "start" is highly synchronous. Provided the subcultures are incubated a minimal length of time at the permissive temperature, sufficiently long to ensure full "start" release, they should all pass through the subsequent rate-limiting step independently of temperature. Since the experiment is stopped when the rate-limiting step is almost completed (80% budding at 25 °C), all the samples should have the same fraction of buds at the end of the experiment, irrespective of the length of incubation at the permissive temperature. The second possibility is that "start" itself is the rate-limiting step. Since the mutants are temperature sensitive for "start", they can emerge at "start" only at the permissive temperature. Therefore, one would expect the fraction of budded cells at the end of the experiment to depend upon the incubation period at the permissive temperature: the longer the period at 25 °C, the lower the fraction of unbudded cells.

In accordance with the second alternative, it was found that the rate of escape from the "start" block followed first-order kinetics identical to the kinetics of bud initiation at the permissive temperature, with a difference of 10 min (Fig. 2). We conclude that "start" is indeed the first-order rate-limiting step of the cell cycle. The 35-min lag period, prior to emergence from "start", may be required for activity of the *cdc25* gene product to allow the realisation of the "start" event. Budding is observed 10 min after this realisation. An essentially similar result was obtained when the initial arrest of *cdc25* cells was by α -hormone (not shown).

Two additional points can be argued in favour of the location of the rate-limiting event at "start". The existence of such a step in the cell cycle should cause accumulation of cells before this step in any growing population. Therefore, the rate-limiting event must be situated in a phase where the trapped cells may stay viable for long periods, as required also from stationary phase cells. Indeed, stationary phase cells accumulate before "start"^{5,6}. The second point is based on the nature of the cell cycle in *S. cerevisiae*. Analysis of *cdc* mutants reveals that after "start" the cell cycle diverges into two independent

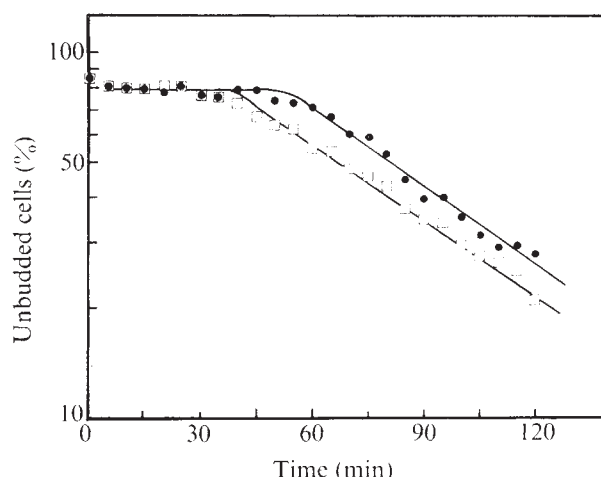


Fig. 2 A comparison between the rate of bud initiation and emergence from "start". Exponentially growing cells of strain 352/1 in liquid YEPD medium were incubated for 3 h at 36 °C. After sonication the cells were shifted to 25 °C. At 5 min intervals samples were fixed with formaldehyde (●), or shifted back to 36 °C and fixed 125 min after zero time (□). Note that the time scale applies directly to budding in the control culture (●) and to times of transfer in the shifted samples (□), where budding was scored at 125 min.

parallel pathways: One is that of DNA synthesis and nuclear division, and the other is composed of bud initiation and nuclear migration⁵. The pathways converge before cytokinesis. The rate-limiting event in the cell cycle must be situated before the divergence of the two pathways because otherwise buds would emerge without nuclear division, or, conversely, multinucleated cells would be formed.

A possible interpretation for the first-order kinetics of cell-cycle initiation is that "start" is a probabilistic event. Although each cell may have the same probability of emerging from "start", only a fraction of the culture would initiate the cycle per unit time. The "transition probability" of each cell, as termed by Smith and Martin⁷, determines the magnitude of this fraction. The stage of accumulation of cells in G_1 prior to "start", can be considered the probabilistic state of the cell cycle (or "A state" of Smith and Martin), in which the cell can spend a variable length of time. The probability element in "start" release does not necessarily have to be the only factor influencing the event. It is likely that the physiological condition of the cell would also affect "start", either by imposing threshold requirements (such as a minimal cell size), or by modifying the rate constant.

It seems that "start" is the only rate-limiting step in the cell cycle of yeast. On initiating the cycle, a cell enters the deterministic phase (or "B phase" of Smith and Martin), which is an orderly sequence of events with a constant length of time. This is indicated by the behaviour of the mutant *cdc24*, which is blocked after "start" and shows synchronous bud emergence following a shift to the permissive temperature (Fig. 1). A second indication of the determinative nature of events following "start" is that bud initiation and emergence from "start" follow identical kinetics in double transfer experiments (Fig. 2). A third indication is that the formation of large buds, which occurs at a later stage of the cell cycle, follows that of small buds at an approximately similar rate (Fig. 3). That no additional rate-limiting step occurs between budding and the next "start" event is also apparent from the kinetics of emergence of second buds following *cdc24* release, which are similar to the kinetics of emergence of first buds in *cdc25* (insert of Fig. 1).

We propose that only a fraction of the cell population

is proliferating. The remainder is waiting in a resting state, equivalent to the stationary phase, and may emerge into the active cell cycle at any time with a fixed probability. Thus the doubling time of the total cell population is expected to be longer than the cycle time of the proliferating cells. The cycle time is defined as the length of the deterministic phase of the cell cycle. It can be estimated as the period between the appearances in the cell population of the first and the second buds on solid medium (Fig. 3). This value (80 ± 5 min) can be a slight underestimate of the mean cycle time because it relates to a fraction of the population that might be faster for physiological reasons (for example, newly formed cells may have a longer cycle time). Doubling time in this experiment (115 ± 10 min) was found to be 1.4 times longer than the cycle time, as expected from our proposition. Other estimates of the cycle time could be used, such as the period between the first and second peaks of small buds in liquid medium (this is an overestimate). These estimates were also found to be smaller than the doubling time.

What are the implications of the rate of cell-cycle initiation on the growth rate of the cell population? Modification of cell-cycle initiation rate by environmental conditions should change the doubling time of the population by changing the average period spent by each cell prior to "start". Indeed, when grown in PSP2 medium, where the doubling time of the population was 1.8 times longer than in YEPD medium, the rate constant of bud emergence decreased significantly (Fig. 4). Doubling time in PSP2 was 1.7 times longer than the period between the first and second buddings on solid PSP2. (PSP2 is an acetate growth medium and YEPD is a glucose medium; see details in Figs 1 and 4). In contrast to these observations with *cdc25*, the rates of bud emergence from a *cdc24*

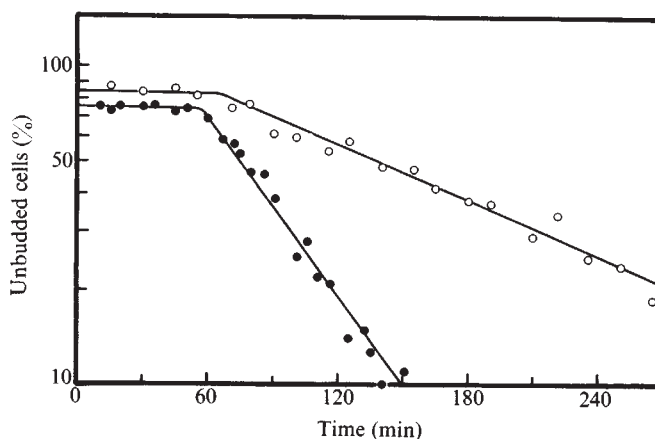


Fig. 4 Rate of bud emergence in two different media. The strain 452/16 was grown in liquid YEPD growth medium (●) or PSP2 acetate growth medium (○), containing 6.7 g yeast nitrogen base, 1 g yeast extract, and 10 g potassium acetate in 1 l of 0.05 M potassium phthalate buffer (pH 5) and supplemented with 40 mg adenine. After 3.5 h at 36 °C the cells were plated on solid media (2% agar) and shifted to 25 °C. Samples were scored as described in legend to Fig. 1. Note: after 7 h on PSP2 solid medium, 2% of the cells remained unbudded.

block in the two different media were similar (not shown). It appears that only the rate of cell-cycle initiation is modified in PSP2.

Another implication of the probabilistic nature of "start" is that it is impossible to synchronise cell populations beyond a certain level by methods employing blocks at "start". Even synchronisation attained by other methods, such as blocks at other stages or selection procedures, would be lost at the first "start" point encountered. Indeed, this has been common experience in yeast synchronisation experiments⁸ and is shown in our experiment with *cdc24* (insert of Fig. 1).

In conclusion we propose that in *S. cerevisiae* a probabilistic event occurs at the cell-cycle initiation, the "start" point, which is also the rate-limiting step of the cycle. The rate of cycle initiation can be altered by changes in the environment, thus modifying the growth rate of the population. The probabilistic event suggested for the mammalian cell cycle¹ may similarly be identified with the main regulatory point of the cycle, the "restriction point". The latter is located at the point of emergence from the resting state in G_1 into the proliferative cell cycle and is analogous to "start".

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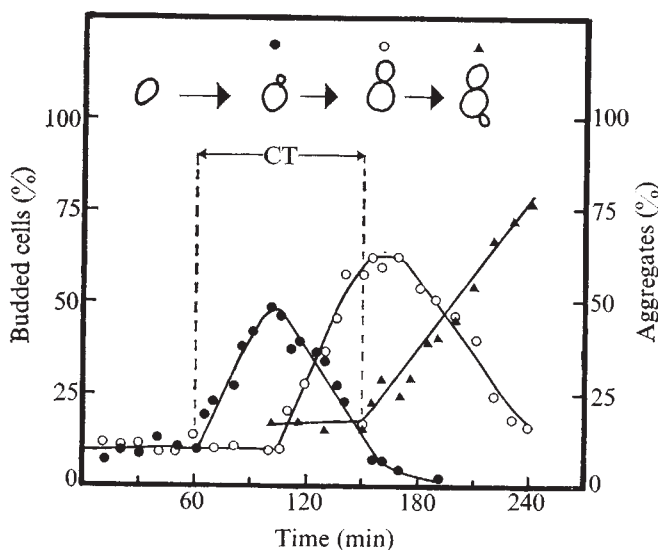


Fig. 3 Kinetics of formation of small buds, large buds and cell aggregates. The strain 452/16 is a diploid, homozygous for the mutation *cdc25*. It is also heteroallelic for *ade2* and carries the following mutations in a heterozygous constitution: *ade1*, *ura1*, *tyr1*, *his7*, *lys2*, *gal1*, *leu*, *trp*, *arg*, *met*, *iso* and *can1*. Exponentially growing cells in liquid YEPD medium were incubated for 3.5 h at 36 °C. After sonication the cells were shifted to 25 °C and plated on YEPD solid medium (2% agar). The percentages of small buds (●), large buds (○) and aggregates of three or more cells (▲) were determined. Small buds were defined as having less than half the diameter of the parent cell, large buds as having greater than half¹⁰. The cycle time (CT) was measured by the period between the appearances in the culture of the first buds and of cell aggregates of three or more cells. Doubling time at 25 °C was determined by counting the cells in a haemocytometer or by viable counts on YEPD agar plates, and was found to be 115 ± 10 min.

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EGTA and proteinase reversal of cellular aggregation of activated lymphocytes

ABUNDANT evidence indicates that cellular aggregation is essential for cellular cooperation in a number of physiological events, including embryogenesis and morphogenesis¹. Cell-to-cell contact has been observed in lymphoid-macrophage cell cultures activated by antigens^{2,3}; furthermore, it is recognised that proliferative responses to mitogenic lectins are dependent upon contact between cells^{4,10}. To date, the nature of the cellular associations in lymphoid-macrophage cultures activated by antigens and mitogens has not been clarified. We designed the present study to evaluate some aspects of cellular associations in lymphoid-macrophage cultures activated by a variety of antigens and the mitogenic lectin, phytohaemagglutinin (PHA). Enhanced cellular aggregation was observed in cultures activated by all agents tested. Time and temperature experiments indicate that aggregation is an active process, distinct from passive agglutination. The cellular association is sensitive to proteinase and the calcium chelator, EGTA, suggesting that both proteins and calcium are essential for the cell aggregation of activated cultures.

All experiments utilised heparinised human peripheral blood sedimented in 5% dextran. The leukocyte-rich plasma was layered onto a ficoll-hypaque gradient and centrifuged at 700g for 45 min. Separated cells were washed three times in Dulbecco's phosphate-buffered saline and resuspended at a final concentration of 6×10^5 cells ml⁻¹ in RPMI-1640 media supplemented with penicillin and streptomycin (Associated Biomedic Systems, Buffalo, New York) and 10% heat inactivated (56 °C for 30 min) single donor human AB+ sera. The appropriate mitogen or antigen was then added to the cells. The cells were placed into wells of microplates (Falcon Plastics) in 0.2 ml volumes and incubated at 37 °C in a humidified atmosphere with 5% CO₂. Cellular aggregation was determined by directly aspirating the contents of the single microplate well with either a Pasteur pipette or a tuberculin syringe fitted with a flat-tipped 19 gauge needle and counting the cell suspension on a haemocytometer. The number of free cells counted, clusters of three or more cells being excluded, was found to be inversely proportional to the amount of cellular aggregation.

In the first series of experiments we determined the degree of cellular aggregation as a measure of cellular association. Results of a typical experiment, plotted in Fig. 1, indicate that purified PHA produces a significant degree of cellular aggregation (as determined by a decrease in the number of free cells). Additional data (not shown) demonstrate that the total number of cells in culture increases during the culture period, indicating that the observed decrease in the number of free cells is not due to a decrease in the total number of cells. As shown in Fig. 1, control cultures also exhibit some degree of cellular aggregation. It should be noted that a purified form of PHA, HA 16/17 (Burroughs-Wellcome), was used in these studies. HA 16/17 is known to have markedly reduced haemagglutinating activity compared to the crude extracts of the mitogen³. In additional experiments (data not shown), aggregation was observed with all mitogenic and antigenic agents tested. These included purified protein

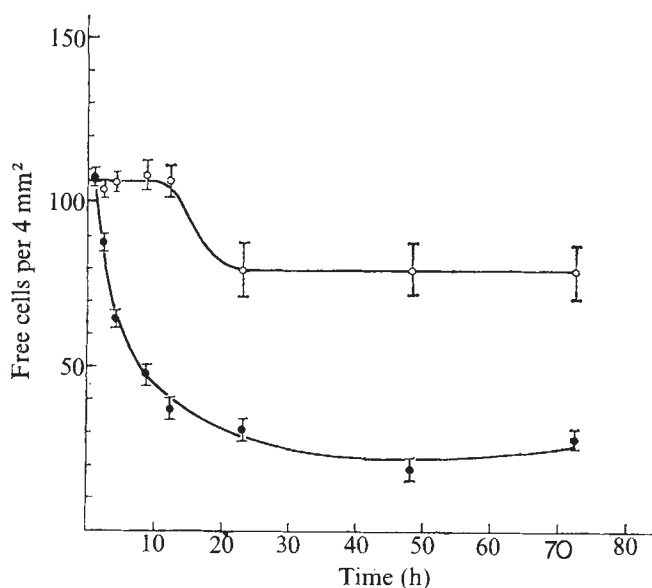


Fig. 1 Cellular aggregation in control (○) and PHA-HA 16/17 (●) treated cultures of Fcoll-Hypaque separated mononuclear cells from human peripheral blood. PHA-HA 16/17 was added to the cultures at a dilution of 1:50. Time after addition of PHA is plotted against number of cells not in aggregates. Each point represents the mean of replicate samples $\pm$ 1 s.d.

derivative of human tuberculin (PPD) (Ministry of Agriculture, Fisheries and Food, Surrey, UK), streptokinase-streptodornase Varidase, (SK/SD) (Lederle Labs), the calcium ionophore A23187 (Eli Lilly, Indianapolis, Indiana), and mixed leukocyte culture using allogeneic lymphocytes. Maximum aggregation was seen on day 6 for PPD, SK/SD, and mixed leukocyte cultures, on day 2 for PHA-HA 16/17, and on day 3 for A23187. It would appear that it is the action of the various activators (that is, the actual activation of the lymphocytes) rather than the presence of the activators that is responsible for the cellular aggregation. If the presence of the mitogens or antigens were sufficient for cellular aggregation, then one would expect to see aggregation earlier than 2 h of culture and perhaps in cultures that have the mitogens and antigens present but are maintained at 4 °C. In neither of these situations did we find aggregate formation.

Several lines of investigation suggest that aggregate formation is an active process which has several requirements: (1) a specific time period, (2) a metabolically active cell, and (3) the presence of calcium. First, aggregate formation initially appears at 2 h of culture with a progressive increase in aggregate formation up to 24 h (Fig. 1). Observation of this aggregate formation process through an inverted microscope revealed a continual increase in first the number and then the size of aggregates during the 24-h period. Second, in no instance do aggregates form when cultures are kept at 4 °C during the first 24 h of culture, regardless of whether the activating agent is

Table 1 Time- and dose-dependent reversal of PHA-HA 16/17-induced cell aggregation by the calcium chelator EGTA

	Free cells per 4 mm ² * (mean $\pm$ 1 s.e.)
No HA 16/17	228 $\pm$ 8
HA 16/17†	36 $\pm$ 2
HA 16/17 + 1 mM EGTA‡	74 $\pm$ 11
HA 16/17 + 5 mM EGTA‡	92 $\pm$ 8
HA 16/17 + 10 mM EGTA‡	121 $\pm$ 11
HA 16/17 + 10 mM EGTA§	160 $\pm$ 28

* Free cells counted after all cells were in culture for 48 h.

† HA 16/17 added at a dilution of 1:50 at the start of culture.

‡ EGTA added 1 h before counting of free cells.

§ EGTA added 2 h before counting of free cells.

PHA, A23187, PPD, or SK/SD. Third, in experiments in which the calcium chelator, EGTA (1–10 mM), is added to cultures at the same time as PHA, the EGTA completely prevents both aggregate formation and DNA synthesis. Thus, this group of experiments suggests that aggregation is distinct from passive mitogen or antigen-induced agglutination.

We next studied the structural basis for aggregate formation by testing the ability of certain agents to dissociate the cell aggregates found in stimulated lymphocyte cultures. Previous work by Moscona and colleagues demonstrated a role for calcium and trypsin sensitive

Table 2 Reversal of SK/SD-induced cell aggregation by EGTA

	Free cells per 4 mm ² * (mean ± 1 s.e.)
No SK/SD	262 ± 12
SK/SD†	63 ± 2
SK/SD + 10 mM EGTA‡	116 ± 6
SK/SD + 10 mM EGTA§	193 ± 24

* Free cells counted after all cells were in culture for 6 d.

† SK/SD added at a dilution of 1:2,000 at the start of culture.

‡ EGTA added 1 h before counting of free cells.

§ EGTA added 2 h before counting of free cells.

macromolecules in the maintenance of aggregates of neural retinal cells⁶. The calcium-specific chelator⁷, EGTA, dissociates aggregated cells in a time- and dose-dependent manner. Table 1 shows the EGTA dissociation of PHA-stimulated cells. Table 2 shows the EGTA dissociation of SK/SD-stimulated cells. Cells aggregated by another mitogen (A23187) and two additional antigens (PPD and allogeneic cells) show similar dissociation with the EGTA. To test whether the EGTA-induced dissociations are due to toxicity or to some nonspecific phenomena, we added CaCl₂ to the EGTA-containing cultures. The dissociation was completely reversed (Table 3), supporting the contention that the EGTA effects are due to calcium chelation. In addition, trypan blue dye exclusion tests indicated that the cells were completely viable. It seemed reasonable, however, that other divalent cations could also be involved in aggregate formation. To test this hypothesis, we incubated cells with EDTA, a chelator with less specificity than EGTA. We observed no additional dissociation from that seen with EGTA alone or from EGTA and EDTA together (data not shown), suggesting that calcium is the major ion necessary for cellular aggregation.

In addition, it seemed possible that the EGTA effects may not be directed at the calcium involved in cell-to-cell binding, but instead on the membrane calcium that is required for maintenance of membrane topography. Cooling lymphocytes to 4 °C is known to significantly reduce ligand-induced movement of surface macromolecules ("cap formation")^{8,9}. Thus, we designed temperature experiments to evaluate the possibility that EGTA directly affects movement of surface macromolecules. Results indicate that cells at 4 °C dissociate with EGTA with only a slight reduction in rate (Table 4), suggesting that EGTA-induced movements in the cell membrane are not the

Table 3 Reversal of EGTA-induced dissociation of PHA-HA 16/17 aggregates by calcium chloride

	Free cells per 4 mm ² * (mean ± 1 s.e.)
HA 16/17†	19 ± 3
HA 16/17 + 10 mM EGTA‡	49 ± 5
HA 16/17 + 10 mM EGTA + 4 mM CaCl ₂ §	14 ± 2

* Free cells counted after all cells were in culture 48 h.

† HA 16/17 added at a dilution of 1:50 at the start of culture.

‡ EGTA added 2 h before counting of free cells.

§ CaCl₂ added 30 min before counting of free cells.

Table 4 Effect of temperature on EGTA dissociation of PHA-HA 16/17-induced cellular aggregation

Duration of incubation (min)	Control	10 mM EGTA
	Cells maintained at 37 °C	
30	14 ± 2*	27 ± 1*
60	15 ± 2	40 ± 2
120	19 ± 2	68 ± 2
	Cells maintained at 4 °C	
30	17 ± 1	21 ± 1
60	15 ± 1	26 ± 1
120	13 ± 0	41 ± 2

* Free cells: mean ± 1 s.e.

major reason for the experimental results.

Finally, we examined the role of proteins in cellular aggregation by adding proteinase to cell aggregates. A typical experiment illustrated in Table 5 shows that trypsin causes aggregate dissociation to a degree quite comparable to that of EGTA.

Table 5 Dissociation of PHA-HA 16/17-induced cell aggregation by trypsin

	Free cells per mm ² * (mean ± 1 s.e.)
HA 16/17†	18 ± 3
HA 16/17 + 0.50% trypsin‡	41 ± 1
HA 16/17 + 10 mM EGTA§	46 ± 2

* Free cells counted after all cells were in culture 48 h.

† HA 16/17 added at a dilution of 1:50 at the start of culture.

‡ Trypsin (1:250, Difco) added 30 min before counting of free cells.

§ EGTA added 2 h before counting of free cells.

In summary, our data suggests that the cellular aggregation produced in lymphoid cultures activated by a variety of antigens and mitogens is an active, time-, energy-, and calcium-requiring process. Trypsin sensitive molecules appear to be involved in cellular aggregation. The molecular nature of the trypsin-sensitive molecule(s) is not clear at present. Our EGTA and calcium reversal data suggest that calcium is directly involved in the binding process. In our experimental conditions, we were unable to demonstrate that other divalent cations are involved in binding. Calcium could be directly involved in the binding process in at least two ways: It is possible (1) that the calcium could be required for maintenance of the three-dimensional structure of the trypsin-sensitive macromolecules involved in cell binding, and/or (2) that calcium, because of its divalent nature, could be involved in bridge formation between cells.

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Activation of alveolar macrophage collagenase by a neutral protease secreted by the same cell

MAMMALIAN collagenase is a proteolytic enzyme which specifically cleaves native triple helical collagen at physiological pH and temperature. As with other extracellular proteases, there is increasing evidence that this enzyme can be found in an inactive, latent form which may represent a proenzyme or an enzyme-inhibitor complex¹⁻⁸. It has been known for some time that, like the zymogen forms of the pancreatic proteases, latent collagenase could be activated with low concentrations of trypsin²⁻⁵. The existence of inactive mammalian collagenase has been perplexing because this is often the major state in which the enzyme is found: it has not been known whether trypsin activation of latent collagenase has any physiological significance. Drawing parallels to the physiological activation of pancreatic chymotrypsinogen, procarboxypeptidase and proelastase by pancreatic trypsin, several investigators have suggested the existence of protease(s) that can activate latent collagenase in the physiological conditions in which collagenase functions¹⁻³. The study reported here validates this concept by demonstrating that a single cell type, the alveolar macrophage, secretes an inactive form of collagenase that can be activated in physiological conditions by a protease secreted at the same time by the same cell type.

Pulmonary alveolar macrophages (PAM), obtained by lung lavage of BCG-treated rabbits, were cultured at 37 °C with daily changes of Dulbecco's modified Eagle's medium (without serum) as previously described⁹. More than 99% of these cells can be shown to be macrophages by their morphology and ability to ingest latex particles⁹. Collagenase activity in the PAM culture media was quantified with a ¹⁴C-collagen fibril lysis assay using neutral-salt-extracted guinea pig ¹⁴C-collagen (40,000 c.p.m. mg⁻¹ collagen)⁹. All assays were performed in conditions in which peptide release was linear with time and concentration of enzyme. Previous studies of the reaction products of PAM collagenase have demonstrated its specificity in cleaving types I and III collagen approximately three-quarters of the distance from the N-terminus of the native collagen⁹. In normal culture conditions, 65–70% of the collagenase produced by PAM was in the active form, with the latent enzyme able to be activated with trypsin⁵.

Table 1 Relationship of the percentage of pulmonary alveolar macrophage collagenase that is in the active form to the neutral protease activity in the same culture medium

Collagenase in active form (%)	Neutral protease activity in culture medium (U ml ⁻¹)
0	0
7	0.2
25	1.8
63	3.2

The percentage of collagenase in active form was calculated from the formula: (collagenase activity in culture medium) × 100 / (collagenase activity in culture medium after activation with trypsin). The different levels of neutral protease activity present in PAM culture medium were modulated by adding BSA in concentrations up to 20 mg ml⁻¹ to the PAM cultures after 24–48 h of incubation. With BSA (20 mg ml⁻¹) all the collagenase in the PAM culture medium was in the latent form and no neutral protease activity was detectable; with no BSA added to the PAM cultures, 65–70% of the collagenase was found in the active form. In these cultures, total collagenase activity was 6–10 µg of collagen lysed per h per 10⁶ cells⁹. Intermediate values demonstrated that the relationship between the percentage collagenase in active form and units (U ml⁻¹) of neutral protease in the culture medium was linear.

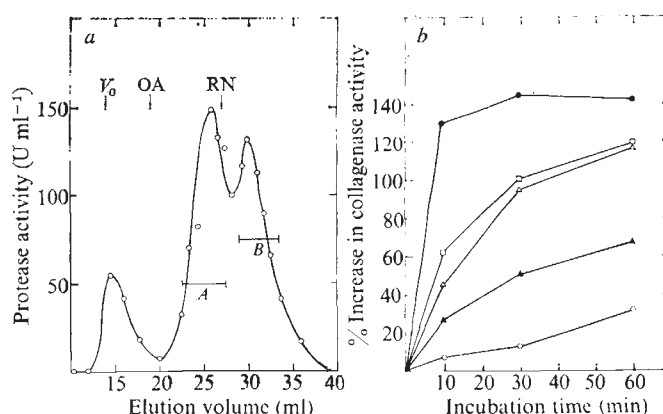


Fig. 1 Gel filtration chromatography of neutral protease activities found in culture media of rabbit alveolar macrophages and the use of these proteases in activating latent PAM collagenase. *a*, PAM culture medium collected between 24 and 48 h was subjected to ultrafiltration through a PM 30 membrane; the filtrate was concentrated with Aquacide II (Calbiochem) and chromatographed on a column (0.9 × 30 cm) of Ultragel AcA54 (LKB, 5% acrylamide–4% Agarose) with 50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂, 200 mM NaCl as the eluting buffer. To determine neutral protease activity, fractions (0.5 ml) were incubated (3 h, 37 °C) in a shaking water bath with 200 µg of ¹⁴C-globin (5 × 10⁴ c.p.m. mg⁻¹), 50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂ and 200 mM NaCl. After incubation, trichloroacetic acid (10%) was added (1 h, 4 °C) to precipitate the intact ¹⁴C-globin. After centrifugation (10,000g, 5 min) the supernatant containing ¹⁴C-peptides was counted in Aquasol. 1 U = digestion of µg of globin per h; this is equivalent to the activity of 15 ng of trypsin in the same assay. Ovalbumin (OA) and ribonuclease (RN) were used as molecular weight standards. Fractions eluting in region A (protease fraction A) and B (protease fraction B) were pooled and concentrated with Aquacide II. *b*, To activate latent collagenase, PAM culture medium concentrated with a PM 30 membrane was preincubated (before collagenase fibril assay) at 37 °C for 0–60 min in buffer (50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂, 200 mM NaCl) plus protease fraction A (▲), protease fraction B (△), PM 30 filtrate protease (concentrated PM 30 filtrate of PAM culture media) (□) or trypsin (5 µg ml⁻¹) (●). Incubation with buffer alone (○) served as a control (no added enzyme). PM 30 filtrate protease, protease fraction A and protease fraction B had no collagenase activity in the ¹⁴C-collagen fibril assay. The amount of each used for collagenase activation had approximately the same neutral protease activity in the ¹⁴C-globin assay. After preincubation, BSA (10 mg ml⁻¹) was added to inhibit the action of the neutral protease on any other proteins in the subsequent collagenase assay; in all cases, this concentration of BSA completely stopped all neutral protease activity present. In the conditions used, it was assumed that preincubation with trypsin would activate all the latent collagenase in the culture medium (6–10 µg collagen lysed per h per 10⁶ cells)⁹. The percentage increase in collagenase activity due to activation by preincubation was defined as: ((collagenase activity after preincubation) – (collagenase activity without preincubation)) × 100 / (collagenase activity without preincubation). Before preincubation, 60% of the collagenase was in the latent form.

Neutral protease activity in PAM culture medium was quantified as the trichloroacetic-acid-soluble ¹⁴C-peptides release from ¹⁴C-globin purified from rabbit reticulocyte haemoglobin labelled with ¹⁴C-leucine¹⁰. The protease assay was performed in conditions in which the release of peptides was linear with time and enzyme concentration.

When preliminary studies demonstrated that PAM cultures with large quantities of neutral protease also had large quantities of active collagenase (and comparably less latent collagenase), methods were developed to evaluate a possible relationship between these enzymatic activities. The addition of bovine serum albumin (BSA) to the PAM cultures was found to be a useful means of varying the relative amounts of neutral protease activity available in the culture media, for the BSA acted as a competitive inhibitor of the action of neutral protease on other proteins while not significantly altering collagenase secretion. When an excess of BSA was added, little protease activity and relatively less

active collagenase was found (Table 1). Alternatively, when no BSA was added, there was more protease activity and relatively more collagenase was in the active form. Thus, there seemed to be a direct relationship between the percentage of the total secreted PAM collagenase that was active and the relative amount of neutral protease activity in the same culture media.

Since the bulk of PAM neutral activity in PAM culture medium was less than 30,000 daltons, partial purification was achieved by ultrafiltration through an Amicon PM 30 membrane and concentration of the filtrate. When this material was chromatographed (Fig. 1a), neutral protease activity was found in three regions; a minor peak in the void volume (>60,000 daltons) and two major peaks, one at 20,000 daltons (protease fraction A) and one at 11,000 daltons (protease fraction B). Peak activities of protease fractions A and B were concentrated and neither were found to have active or latent collagenase activity. The activity of these fractions against the ^{14}C -globin substrate was inhibited by 10 mM ethylenediaminetetraacetate, BSA (10 mg ml $^{-1}$) or 5% serum but not by 2 mM phenylmethylsulfonylfluoride, α_1 -antitrypsin (100 $\mu\text{g ml}^{-1}$) or 10 mM *N*-ethylmaleimide.

One possible physiological role of these protease fractions is demonstrated in Fig. 1b, which shows that latent collagenase from PAM culture medium was activated in physiological conditions by preincubation (before the collagenase assay) with the neutral protease(s) secreted by the same cells. Preincubation with protease fraction A increased collagenase activity by 65% while protease fraction B increased collagenase activity by 120% (which was 90% that achieved with trypsin). The activation of latent collagenase by protease fraction B was the same as that achieved with partially purified (PM 30 filtrate) protease. Evidence that the neutral protease(s) truly activated latent collagenase (as opposed to appearing to "activate" by directly attacking the ^{14}C -collagen substrate in the collagenase assay) came from two observations: (1) neither fraction A or B had collagenase activity in the fibril assay, and (2) at the end of preincubation, sufficient BSA was added to inhibit all neutral protease activity. Further evidence for the role of neutral protease(s) in activating latent collagenase came from the observation that while preincubation at 37 °C for 1 h with buffer alone caused a 30% increase in collagenase activity, this phenomena (so called "autoactivation") was obviated by including BSA with the buffer.

The secretion of degradation enzymes in an initial precursor form is well documented¹¹. It is, however, often difficult to distinguish a true zymogen, in which a portion of the precursor enzyme must be removed to activate the enzyme, from an enzyme-inhibitor complex, from which the inhibitor must be removed or inactivated to activate the enzyme. For collagenase, there is argument for both possibilities^{1-8, 12-14}. For the PAM collagenase, gel filtration of completely latent collagenase demonstrates the bulk of activatable collagenase chromatographs at approximately 49,000 daltons (Fig. 2). When this partially purified latent collagenase was activated with protease B before chromatography it was found at an apparent molecular weight of 31,000. Thus, there is direct evidence of the conversion of a latent collagenase to an active collagenase of lower molecular weight by a neutral protease secreted by the same cell. There is no possibility that the 49,000-dalton form of the enzyme is a collagenase-collagen complex, since the rabbit PAM does not synthesise collagen (unpublished observations of N. A. Elson and R.G.C.). These data, however, do not answer further the question of whether the latent collagenase is a true zymogen or an enzyme-inhibitor complex.

Activators of latent collagenase have been suggested before: for example, tadpole culture fluid activation of tad-

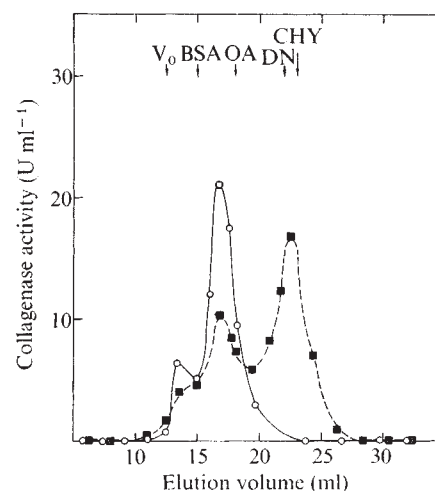


Fig. 2 Gel filtration chromatography of latent and active rabbit PAM collagenase. PAM were cultured in medium containing BSA (10 mg ml $^{-1}$). The medium was collected after 0–24 h of incubation and concentrated by Amicon PM 30 membrane ultrafiltration. The resulting material contained no neutral protease activity and 100% of the collagenase was in the latent form. Chromatography was performed on Ultragel AcA54 (LKB, 5% acrylamide–4% Agarose) eluted with 50 mM Tris-HCl, pH 7.4, 5 mM CaCl $_2$, 200 mM NaCl. Fractions (0.5 ml) were collected and assayed for collagenase activity using the ^{14}C -collagen fibril assay after trypsin activation (○) (50 μl samples of each fraction were incubated with trypsin (10 $\mu\text{g ml}^{-1}$) for 20 min at 23 °C followed by incubation with soybean trypsin inhibitor (50 $\mu\text{g ml}^{-1}$) for 10 min). In the ^{14}C -collagen fibril assay, 1 U = 1 μg of collagen lysed per h. Separate chromatography of molecular weight standards (BSA), ovalbumin (OA), desoxyribonuclease I (DN) and chymotrypsinogen A (CHY) were used to estimate the major peak of latent collagenase (fractions 16–17) at 49,000 daltons. When the PAM medium was preincubated (37 °C, 45 min) with 120 U of protease B (■) before chromatography, the major peak of collagenase activity had a molecular weight of approximately 31,000. The collagenase activity at the void volume (fractions 13–14) probably represents aggregated enzyme¹⁵; rechromatography of this larger molecular weight form in a higher ionic strength buffer (1.2 M KCl compared with 0.2 M NaCl) caused part of the collagenase activity in this large molecular weight region to shift to the major peak of collagenase activity.

pole tail collagenase¹; human rheumatoid synovial fluid activation of human granulocyte collagenase⁶; human microbial plaque activation of human gingival collagenase⁸; and mouse liver cathepsin, hog pancreatic kallikrein or human serum plasmin activation of mouse bone collagenase⁷. Our data suggest that at least for the alveolar macrophage, the same cell type secretes collagenase in a latent form, as well as protease(s) which activate it. Both (collagenase and neutral protease) activities are demonstrable in physiological conditions and there is a correlation between protease activity and relative collagenase activation. This finding helps to explain the phenomena of collagenase "autoactivation" whereby unpurified preparations of collagenase can be activated by incubation at 37 °C, a finding that is often exaggerated when collagenase-containing culture media are concentrated. Most importantly, this demonstration of interactions between protease and latent collagenase gives a physiological explanation for *in vitro* trypsin activation of latent collagenase and suggests important control points in the regulation of collagen degradation.

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Vasopressin-induced redistribution of binding sites for concanavalin A at the surface of epithelial cells from urinary bladder

VASOPRESSIN promotes water movement across the epithelium of the distal portion of mammalian renal tubule and amphibian urinary bladder^{1,2}. The hormone also elicits a general, non-selective increase in the transcellular permeation of lipophilic solutes³ and a decrease in the discrimination between straight- and branched-chain isomers^{4,5}. Such changes in the permeability of amphibian urinary bladder are limited to the apical membrane of those epithelial cells rich in intracellular secretion granules^{6,7}. These granules, some of which may correspond to lysosomes⁸, are observed to migrate to the mucosal membrane of toad bladders treated with oxytocin or cyclic AMP⁹. Moreover, treatment with vasopressin provokes the extracellular release of acid hydrolases at the apical surface of cells from bullfrog bladder¹⁰. Additional evidence shows that repression of such hydrolase activity at the external surface reduces the hormone-induced water flow observed across the intact epithelium⁷. My present investigation extends these observations by providing direct evidence in epithelial cells from bullfrog bladder for vasopressin-induced rearrangement into clusters of binding sites for concanavalin A (con A) which are otherwise randomly disposed at the surface of control cells. This hormone effect is inhibited by prior incubation of cells with the microtubule-disrupting drug, colchicine. In addition, colchicine treatment antagonises the enhancement of extracellular hydrolase release and transepithelial water flow attributable to vasopressin action.

Epithelial cells were isolated from the urinary bladders of *Rana catesbeiana* (Mogul-Ed, Oshkosh, Wisconsin) by procedures described previously⁷. The cells were maintained at 22 °C and suspended in Ringer solution constituted as follows: 104.5 mM NaCl, 2.7 mM KCl, 0.5 mM CaCl₂, 0.6 mM MgSO₄, and buffered at pH 7.4 with 2.125 mM Na₂HPO₄/0.375 mM NaH₂PO₄. Aliquots (2 ml; approximately 3 × 10⁶ cells) were incubated in the presence or absence of test reagents and appropriate control compounds for selected times in a Dubnoff shaking incubator. Epithelial cells in the several experiments were exposed to arginine vasopressin (AVP), chlorobutanol, colchicine, α-methyl-D-mannoside (all from Sigma), con A (Calbiochem), or combinations of these agents. Colchicine was converted to biologically inactive lumicolchicine by irradiation with ultraviolet light as described by Wilson and Friedkin¹¹. Cell viability was evaluated by the method of dye exclusion¹². At least 95% of all cells used here excluded the dye, Trypan blue, at a final concentration of 0.05% during 10 min incubation in Ringer solution at 22 °C.

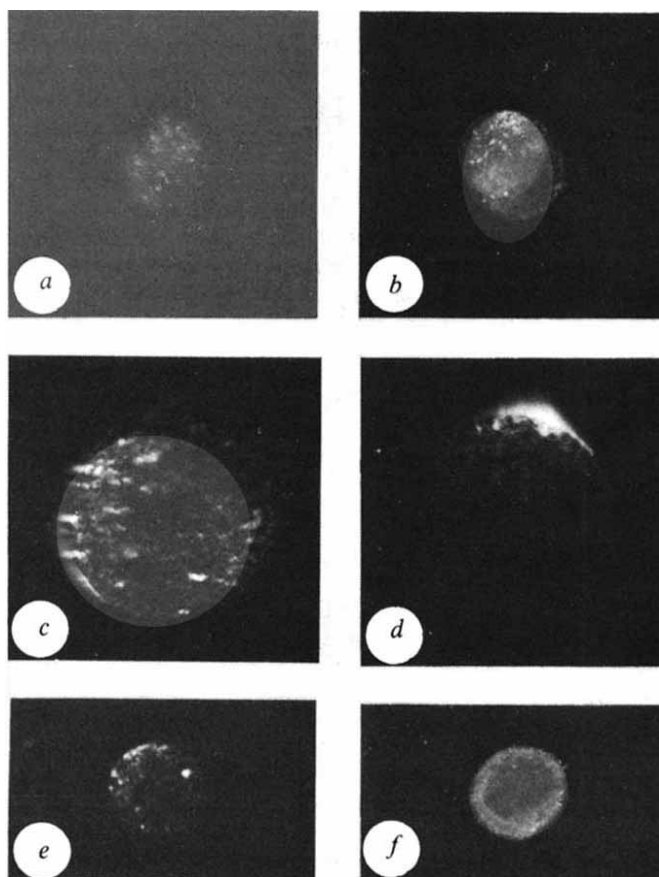
Previous studies have shown that epithelial cells isolated from bullfrog urinary bladder possess specific binding sites for fluorescein-labelled con A (F-con A). Cells bind the lectin maximally at a concentration of 50 μg F-con A ml⁻¹

when incubated for 3 min at 22 °C (ref. 13). These conditions were used to evaluate, by dark-field ultraviolet-fluorescence microscopy, F-con A binding to cells that had been incubated with or without 10 mU AVP ml⁻¹ for 15 min.

F-con A was distributed in apparently random fashion over the surface of the majority of cells treated only with hormone vehicle, 5.6 × 10⁻¹¹ M chlorobutanol. A representative example is presented in Fig. 1a. Diffuse clusters of label were however, occasionally present at the surface of control cells (Fig. 1c). Addition of AVP to cells for 15 min evoked a marked concentration of fluorescence at one pole of the cell surface in approximately 45% of all isolated cells observed (Fig. 1b, d). Moreover, many of those cells treated with hormone tended to bind together, with aggregates of F-con A evident at the points of cell contact. As in previous experiments with epithelial cells from urinary bladder¹³, binding of F-con A at the cell surface could be blocked by the addition of 0.1 M α-methyl-D-mannoside or excess unlabelled Con A during the incubation of cells with F-con A (not shown).

In view of recent reports that indicate that the microtubule-disrupting drug, colchicine, counteracts the hydrosmotic effect of vasopressin in toad urinary bladder¹⁴, the

Fig. 1 Dark-field fluorescence micrographs of F-con A binding to epithelial cells from bullfrog urinary bladder. Cells were incubated in the absence (Fig. 1a, ×400; Fig. 1c, ×1,000) or presence (Fig. 1b, ×400; Fig. 1d, ×1,000) of 10 mU AVP ml⁻¹ for 15 min as described in the text. Additional cells were incubated for 3 h with 2 × 10⁻⁵ M lumicolchicine (Fig. 1e, ×400) or 2 × 10⁻⁵ M colchicine (Fig. 1f, ×400) before exposure to hormone for 15 min. Photomicrographs were obtained with a Leitz ortholux microscope used in conjunction with a xenon light source, XBO 150. The exciting filter combination consisted of a BG 38 heat barrier filter, a Leitz 480 nm filter and a Leitz KP 490 interference filter; a K 510 nm filter served as the barrier filter. Photographs were taken on Kodak Tri-X pan film (ASA 400). Approximately 250 cells in each treatment group were observed in each of four to six independent experiments.



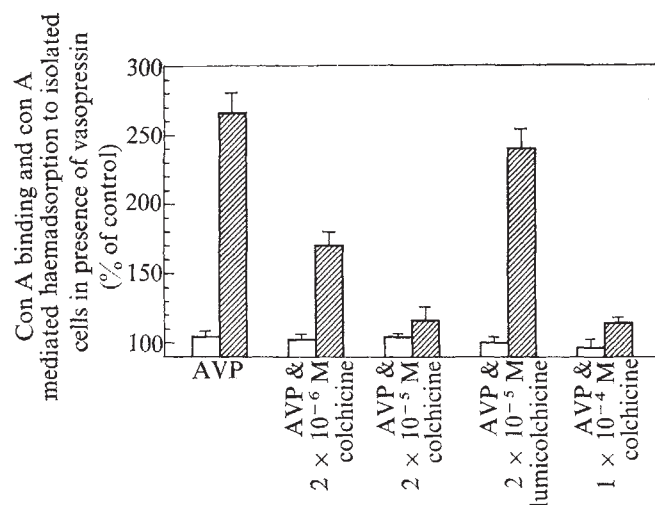


Fig. 2 Effects of prior incubation with various concentrations of colchicine or lumicolchicine (3 h) on F-con A binding (clear bars) and con A-mediated haemadsorption (shaded bars) to epithelial cells in the presence and absence of 10 mU AVP per ml. In methods described previously¹³, maximal F-con A binding to cells was detected at 50 μ g lectin per ml (3 min, 22 °C). With a spectrophotofluorometric value of 6 net fluorescence (arbitrary units) equivalent to approximately 0.10 μ g of con A, lectin binding to the present cells in the absence of added reagents averaged 12.2 ± 0.7 net fluorescence per mg cell protein. Adsorption of isologous erythrocytes to bladder cells was determined from measurements of haemoglobin absorbance (418 nm) associated with filtered/solubilised epithelial cells previously exposed to con A as described before¹³. The basal level of con A-mediated haemadsorption, expressed as absorbance per mg epithelial cell protein, averaged 0.024 ± 0.004 . Treatment of epithelial cells with colchicines alone did not significantly influence these baseline levels of lectin binding or lectin-mediated haemadsorption to cells. The significance of differences between treatment and control preparations was estimated by paired analyses with the Student's *t* test. All experimental values were calculated as mean $\pm$ s.e.m. and are presented as percentage of appropriate controls.

surface distribution of F-con A was examined in the presence and absence of hormone following 3 h prior incubation with colchicine or its biologically inactive derivative, lumicolchicine. As in controls not treated with hormone or drugs, distribution of lectin-binding sites was random at the surfaces of cells exposed only to colchicine or to lumicolchicine (not shown). Addition of hormone to cells incubated with lumicolchicine evoked the characteristic polar redistribution of con A binding sites (Fig. 1e). In contrast, the orientation of label remained diffuse at the surfaces of colchicine-treated cells despite the presence of AVP (Fig. 1f).

A quantitative estimate of lectin-mediated cellular agglutinability in the presence and absence of AVP was obtained by a haemadsorption method previously described¹³. In confirmation of earlier observations¹³, treatment of epithelial cells of bullfrog bladder for 15 min with AVP elicited a marked increment in con A-mediated haemadsorption ($P < 0.001$), but not F-con A binding ($P > 0.90$), as compared to the corresponding values for control cells not exposed to hormone (Fig. 2). This enhancement of the lectin-mediated cellular agglutinability by hormone treatment correlates with the AVP-induced redistribution of F-con A binding sites at the cell surface as described above.

As shown in Fig. 2, epithelial cells treated with AVP after incubation with lumicolchicine continued to show a significant enhancement of con A-mediated haemadsorption ($P < 0.001$), but not lectin binding ($P > 0.90$), as compared to corresponding cells not exposed to hormone. However, treatment of bladder cells with colchicine before addition of AVP evoked a substantial reduction in the expected level of lectin-mediated haemadsorption ($P > 0.05$), but not lectin binding ($P > 0.90$), to cells as compared to that of appropriate

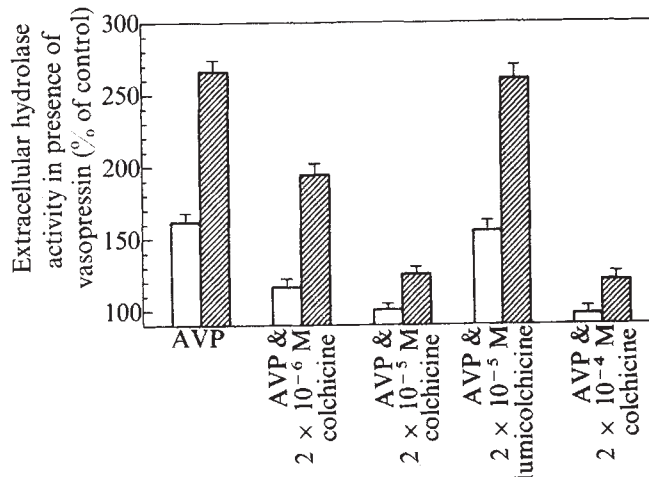
control cells (Fig. 2). The decline in hormone responsiveness was proportionate to the medium concentration of colchicine (Fig. 2).

The influence of colchicine on the AVP-induced release of acid hydrolases from isolated epithelial cells^{7,10} was investigated in further experiments shown in Fig. 3. Treatment of cells with 10 mU AVP per ml alone for 7.5 min enhanced the extracellular activities of cathepsin B1 and acid phosphatase to 266% and 162% of the levels associated with control cells, respectively. Prior treatment of cells with 2×10^{-5} M lumicolchicine for 3 h before exposure to AVP elicited no significant alteration in the hormone-dependent elevation of extracellular hydrolase activities as compared to control cells not treated with AVP (both at $P > 0.90$). But these normal increments in extracellular enzyme activities were markedly reduced among epithelial cells incubated with colchicine at concentrations ranging from 2×10^{-6} to 1×10^{-4} M for 3 h before exposure to AVP.

In view of the above evidence for inhibition of actions of AVP in isolated cells by colchicine, analyses were undertaken of the influence of the drug on hormone-enhanced water permeation across intact epithelium derived from bullfrog. As shown in Fig. 4, treatment of intact bladder with AVP for 1 h elicited an average increment of 600% in water permeability as compared to that of control tissues after prior incubation with lumicolchicine at concentrations ranging from 2×10^{-7} to 1×10^{-4} M (all at $P < 0.001$). However, treatment of bladders with colchicine before addition of hormone markedly reduced the expected increase in water movement attributable to hormone. The maximal inhibition of AVP-induced water flow was observed at a colchicine concentration of approximately 2×10^{-5} M.

The results of these studies show that AVP treatment of epithelial cells isolated from urinary bladder provokes a marked rearrangement of membrane binding sites for con A at the external surface. This response to hormone occurs in the absence of significant alterations in specific con A binding to cells. Moreover, AVP-induced redistribution of

Fig. 3 Effects of prior incubation with various concentrations of colchicine or lumicolchicine (3 h) on extracellular release of acid phosphatase (clear bars) and cathepsin B1 (shaded bars) from epithelial cells treated with and without 10 mU AVP per ml. The activities of cathepsin B1 (EC 3.4.22.1) and acid phosphatase (EC 3.1.3.2) were determined on aliquots of particle-free supernatant fractions collected from the incubation media of isolated cells after 7.5 min treatment with or without hormone as described previously^{7,10}. With extracellular enzyme activities expressed as pmol substrate utilised per min per mg cell protein, basal activities of cathepsin and phosphatase averaged 38 ± 3 and $1,030 \pm 24$, respectively. Treatment of cells with colchicines alone did not significantly alter these control values for extracellular enzyme activity. All experimental determinations are presented as percentage of appropriate controls.



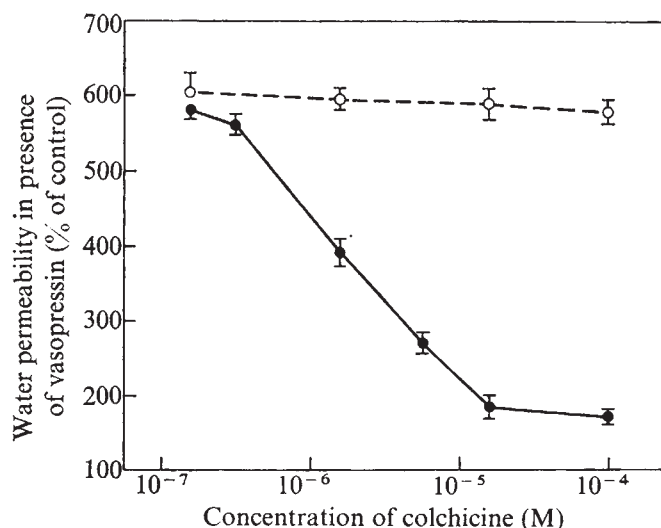


Fig. 4 Effects of prior incubation with various concentrations of colchicine (●) or lumicolchicine (○) on AVP-induced water permeation across bullfrog urinary bladder. Colchicines were present for 3 h in mucosal and serosal media before serosal addition of 10 mU AVP per ml. Radioactive tracer techniques were used to study the movement of water in the mucosal to serosal direction across bladders mounted as flat sheets between two Lucite chambers as described previously³. Permeability measurements were made during the 60-min period following addition of AVP or hormone vehicle. Basal water permeation was corrected for the presence of unstirred layers (170 μ m) as before^{3,7} and averaged $1,200 \pm 100 \times 10^7$ cm s⁻¹. Treatment of bladders with colchicines alone did not significantly influence the water permeability coefficient as compared to paired tissues not exposed to colchicines. All experimental values are presented as a percentage of appropriate paired controls.

lectin-binding sites, as indicated by fluorescence microscopy, may contribute to the enhancement of con A-mediated cellular agglutination¹³. Clustering of lectin-binding sites and the resultant increase in agglutinability of transformed cells has been considered to be a consequence of increased lateral movement of antigen-binding components in the plane of the membrane^{15,16}.

The findings likewise correlate with other evidence for redistribution of intramembranous particles associated with apical membrane of urinary bladder after exposure to neurohypophyseal hormones^{17,18}. In addition, the mobility of spin-label and fluorescent probes and the movement of non-electrolytes in the lipid phase of several cell types are known to increase in response to addition of the appropriate polypeptide hormone^{4,5,19-21}. Such increments in the motional freedom of membrane molecules attributable to hormone may contribute to the aggregation of lectin-binding sites observed in this work.

It has been shown that disruption of microtubules by colchicine treatment reduces AVP-induced water flow across amphibian urinary bladder¹⁴, a finding verified here. This drug effect is accompanied by a marked reduction in lectin-mediated cellular agglutination and in extracellular hydrolase release which normally occurs in response to AVP exposure. Microscopic observation of the distribution of F-con A at the external surface demonstrates that AVP-induced movement of lectin-binding sites is inhibited in cells treated with colchicine, but not lumicolchicine, before exposure to hormone. Thus, it seems that cellular components sensitive to colchicine are essential to the AVP-induced redistribution of lectin receptors at the bladder cell surface. This seems inconsistent with the conclusion that colchicine-sensitive structures constrain the movement of recognition sites for lectins²² and antibodies²³ at the external surfaces of leukocytes. Other studies indicate however that, under some circumstances (such as phagocytosis), colchicine-binding proteins not only serve to anchor surface

elements but are also required for their directed movement²⁴⁻²⁶. The specific displacement of con A binding sites from their random distribution into clusters after AVP exposure, as shown here, likewise seems to require coupling with intracellular elements sensitive to disruption by colchicine.

As suggested from the results of previous studies^{7,9,10,14}, microtubules may participate in the action of AVP on water flow across urinary bladder through their involvement in the mechanism of release of granular enzymes at the apical surface of epithelial cells. Clearly, additional work is necessary to determine the functional significance of this early vasopressin effect on cellular hydrolase distribution. The present report provides additional support for the view that ensuing hormone-induced alterations in the orientation of membrane proteins and in the degree of order or flow of membrane lipids may underlie changes in epithelial transport functions due to vasopressin treatment.

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Antigen-specific helper factors in rabbit lack both V and C region Ig determinants

THE nature and genetic origin of the T lymphocyte antigen-recognition system is an intriguing and compelling immunological problem. T cells differ from B cells in not synthesising classical antibody and in not expressing a readily detectable immunoglobulin (Ig) as their surface receptor for antigen¹. It has recently been found, however, that in some cases T cells and serum antibody carry the same idiotypic determinants²⁻⁴, inferring identity between the binding sites, that is the variable regions, of the T cell antigen receptor and immunoglobulin. It has also recently been discovered that T cells can produce antigen-specific molecules or factors which carry determinants of the major histocompatibility complex (MHC) in addition to a binding site for antigen⁵⁻⁸. The existence of the specific T cell factors raises the possibility of variable (V) region genes

separate from those contributing to antibody and B cell receptors, and perhaps located in the MHC⁹. In order to resolve these possibilities we have attempted to produce antigen-specific T cell factors in the rabbit. The rabbit is the ideal animal for such a study, since it uniquely carries allotypic markers in the V region of its immunoglobulins (see reviews in refs 10 and 11). The sharing of such a V-region allotypic marker by both Ig and the specific T cell factor would establish a common genetic basis for antigen recognition in the two molecules. The results below show, however, that the Ig V region allotypes determined by the *a* locus and indeed other Ig markers are not present on the specific T-cell helper factor in the rabbit and imply the existence of separate V genes for antibody and T cell factors.

The antigen-specific helper factor was obtained from the peripheral blood lymphocytes of rabbits immunised with sheep erythrocytes (SRBC). In testing the factors, advantage was taken of the ability of the specific helper factors to collaborate with B cells across a species barrier. It has been previously shown that the mouse T cell factor will interact with and trigger human lymphocytes in culture^{12,13}. In the experiments described here, the rabbit factor was tested for its ability to cooperate with mouse B cells in a response to SRBC when transferred into irradiated mice. In detail, rabbits were inoculated intravenously with 0.1 ml packed SRBC. Four days later, heparinised blood was taken and erythrocytes removed by sedimenting at 1g in 3% gelatin at 37 °C. Peripheral blood lymphocytes, without further separation, were washed and suspended at a density of 5×10^6 cells ml⁻¹ in RPMI 1640 (Microbiological Associates) containing glutamine and 10% foetal calf serum (Gibco). The cells were incubated under CO₂ at 37 °C in 50 mm Petri dishes for 6–8 h, together with antigen, that is, 0.1 ml 0.1% SRBC per ml of culture. At the end of incubation cell viability was usually greater than 80%. The cells were removed by centrifugation and the culture supernatant was used as the source of the rabbit factor. Its activity was assayed by mixing together with mouse (C57BL/10ScSn) bone marrow cells and antigen (SRBC) and transferring into irradiated (700 R) syngeneic mouse recipients (2×10^7 bone marrow cells, 0.1 ml 10% SRBC per recipient). The plaque forming cell (PFC) response in the recipients was measured 8–10 d after transfer and compared with that in appropriate control groups. The rabbits used were homozygous and heterozygous at the *a* and *b* loci. The *a* locus determines allotypes of the V region of the heavy chains, while the *b* locus allotypes are present on the κ light chains (see refs 10 and 11). The antiallotypic antisera were raised by a method published earlier¹⁰. Goat anti-rabbit Fc was raised against purified rabbit IgG Fc and absorbed with the Fab fragment. All the antisera were used as immunoadsorbents by coupling the 18% Na₂SO₄ precipitated fraction to activated Sepharose¹⁴.

The results in Table 1 show that primed rabbit peripheral blood cells on incubation with antigen can release a factor which cooperates with mouse bone marrow cells. In the presence of the rabbit factor, the mouse B cells proceed to an IgM anti-SRBC response which would otherwise require the presence of mouse T cells (Table 1). The rabbit factor is specific in its effect and will not cooperate in a response to horse RBC. Further, the factor is absorbed out by packed

Table 2 Effect of anti-Ig adsorbents on rabbit helper factor for SRBC

Immunoadsorbent	PFC [‡]
—	3,200 ± 365
Anti-a [†]	2,450 ± 280
Anti-b [†]	4,900 ± 390
Anti-Fc	3,000 ± 323
Control*	200 ± 26

* Bone marrow cells alone.

[†] The rabbit used in this experiment was homozygous a1 and b4.

[‡] Geometric means ± s.d.

SRBC (not shown in Table 1), presumably by virtue of possessing an antigen binding site. We have not yet sought proof that the rabbit factor is a product of rabbit T cells, although the obvious similarity with the antigen-specific mouse factor makes this very likely. It is also noteworthy that not all rabbits tested produced the factor; the reasons for this may be technical, but are worth closer scrutiny. The ability of the rabbit factor to act in the mouse is very reminiscent of the xenogeneic reaction of mouse T cell factors with human lymphocytes^{12,13} and again underlines that the restrictions on cellular interactions¹⁵ do not apply to the helper factors.

In order to detect Ig V and C region determinants on the rabbit factor, the supernatants of the rabbit cell cultures were passed through Sepharose immunoadsorbents carrying anti-a, anti-b or anti-Fc antibodies. The ability of these adsorbents to remove appropriate rabbit Ig was established. However, the results, of which Table 2 is typical, showed that none of the anti-Ig adsorbents tested was able to bind the rabbit helper factor, since no reduction in helper factor activity was observed after passage through the adsorbents. Partial removal of the factor by the immunoadsorbents would have been detectable if in excess of 50%. Hence the rabbit factors, though able to bind antigen, apparently do not carry the V region *a*-allotypes, the κ light chain *b* allotypes or determinants of the constant region of the γ chain. It may be noted that the expression of the *a*-allotypes on rabbit heavy chains is not dependent on the presence of a light chain.

These results indicate that the helper activity released by rabbit lymphocytes is not due to classical serum antibody. It has already been shown in the mouse that T cell derived antigen-specific helper and suppressor factors lack the determinants of the constant regions of both heavy and light chains^{3,7}. The present work shows that, in addition, the analogous molecules in the rabbit lack the allotypic markers associated with the V regions of the majority of circulating Ig molecules. We would propose that this result also holds true for the T cell surface receptor for antigen, since it is very unlikely that the V region of the T cell factor would be different from that of the T cell receptor. It should be noted that a small proportion (10%) of antibody molecules in the rabbit also lack the *a* locus allotypes (see refs 10, 11) and we cannot rule out the possibility that the factors share V regions with this minority Ig population. Taken together with molecular studies of the antigen-specific helper factor in the mouse^{8,9,12}, however, these findings would tend to support the existence of an Ig-independent V-gene pool for T cell recognition. Since the mouse factor is at least partially coded by genes in the I region of the mouse H-2, the T cell V gene pool might also be expected to be associated with the major histocompatibility complex⁸.

Our result seems to be at odds with those of other workers who have demonstrated the presence of antibody idiotypes on T cells specific for histocompatibility antigens², phosphorylcholine⁴, and streptococcal carbohydrate³. Genetic linkage for some of these shared idiotypes to Ig allotypes has been established in the mouse¹⁶, though the possibility that the idio-type-bearing molecule is passively acquired from serum has not been ruled out in all cases¹⁶. It could be

Table 1 Activity of a rabbit helper factor for SRBC

Cells and factors transferred	PFC response*
Bone marrow + SRBC	180 ± 25
Bone marrow + thymocytes + SRBC	6,300 ± 520
Bone marrow + rabbit factor + SRBC	3,650 ± 390
Bone marrow + rabbit factor + HRBC [†]	250 ± 22

* Geometric means ± standard deviation.

[†] HRBC, horse red blood cells.

argued that the a allotypes, in contrast with idiotypes, are primarily a marker of the framework of the V region. The sharing of idiotypic determinants by T cell receptors and antibody might then be explained by a mechanism in which Ig hypervariable regions in the form of episomes are inserted into a non-Ig T cell V framework, on the lines of a suggestion already put forward for the antibody itself¹⁷⁻¹⁹. In this case, the T cell factors, while lacking Ig V-region allotypes, could nevertheless carry Ig idiotypic determinants. It remains to be established whether the T cell factors themselves carry Ig idiotypes, and this possibility is presently being investigated.

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Specific unresponsiveness to transplantation antigens induced by auto-immunisation with syngeneic, antigen-specific T lymphoblasts

AUTOIMMUNE reactions have hitherto been considered to be harmful or at best neutral for the individual. Specific autoimmune reactions in certain experimental systems may, however, have consequences of potential positive value for the individual¹. Here, advantage was taken of the fact that antigen-binding receptors of B and T lymphocytes belong to the classes of self-molecules against which autoimmunity can be inflicted²⁻⁴. Such receptors can be classified according to specific antigen-binding capacity or unique antigenic, idiotype markers⁵. In one system studied, autoanti-idiotypic antibodies were induced against rat T and B cell receptors specific for major histocompatibility antigens of the species¹.

Soluble antigen-binding receptors were purified using anti-idiotypic immunosorbents. Autoimmunisation with purified, polymerised receptors lead to production of autoanti-idiotypic immunity. This caused a selective elimination of lymphocytes carrying the relevant idiotype, antigen-binding receptors. Consequently, such autoimmune rats could be shown to be tolerant to transplantation of skin from the proper foreign donor, whereas reactivity against third party skin was left intact. No negative side-effects were noted during immunisation. Thus, autoimmunisation against the individual's receptors for a given antigen may constitute an efficient way of producing specific immune tolerance in adult, immunocompetent individuals. This approach would have obvious possible applications in clinical transplantation immunology, in treatment of allergic disorders, and might even be used when trying to regulate the course of certain autoimmune diseases. The requirement to have accessible anti-idiotypic antibodies to make the necessary immunosorbents to obtain pure receptors, is, however, a severe drawback. An approach allowing autoanti-receptor immunisation without having to rely on sophisticated or unobtainable reagents would be of great usefulness. Here, we report on a technique whereby autoanti-receptor immunity leading to specific immune unresponsiveness can be induced *in vivo* using as immunogen the individual's own antigen-specific lymphoblasts as obtained after *in vitro* immunisation.

The rationale behind the present approach reads as follows. Lymphocytes carry on their surface idiotype, antigen-binding receptors signifying their potential immune reactivity. To obtain the particular subpopulation carrying receptors with specificity for a given antigen in a pure form two steps of purification had to be used. Whereas conventional immunosorbents made up of antigen can be used to selectively purify B lymphocytes, this approach is considerably less efficient for T cells⁶. When lymphocytes respond to immunogen they frequently, however, transform into larger cells, lymphoblasts. Such blasts can be selectively purified from non-responding small lymphocytes using Ig velocity sedimentation⁷. Purified lymphoblasts carry select reactivity against the relevant antigens only, and represent a highly enriched population of cells carrying receptors with the expected antigen-binding, idiotype-positive markers^{8,9}. Such blasts could thus be considered to represent a high local concentration of "pure" idiotype receptor molecules suitable for autoimmunisation if administered in an immunogenic form. To increase the immunogenicity of such blasts when used for autoimmunisation we emulsified the cells in Freund's complete adjuvant before inoculation. The results to be presented indicate that this immunisation procedure will lead to specific autoimmunity and selective abrogation of reactivity against the relevant antigens.

The antigens used for study were the major histocompatibility complex locus antigens of the mouse and rat. T lymphoblasts were obtained from mixed leukocyte cultures using procedures already described^{8,12}. CBA/H blasts obtained after responding to DBA/2, C57BL/6 cells, or Lewis rat T blasts reacting against DA cells were used respectively. After the final Ig velocity sedimentation procedures⁷, 10⁷ blasts or small, non-responding lymphocytes from the same MLC were emulsified in Freund's complete

Table 1 Specific suppression of MLC response to DBA/2 antigens induced by autoimmunisation of CBA/H mice with autologous anti-DB A/2 T blasts

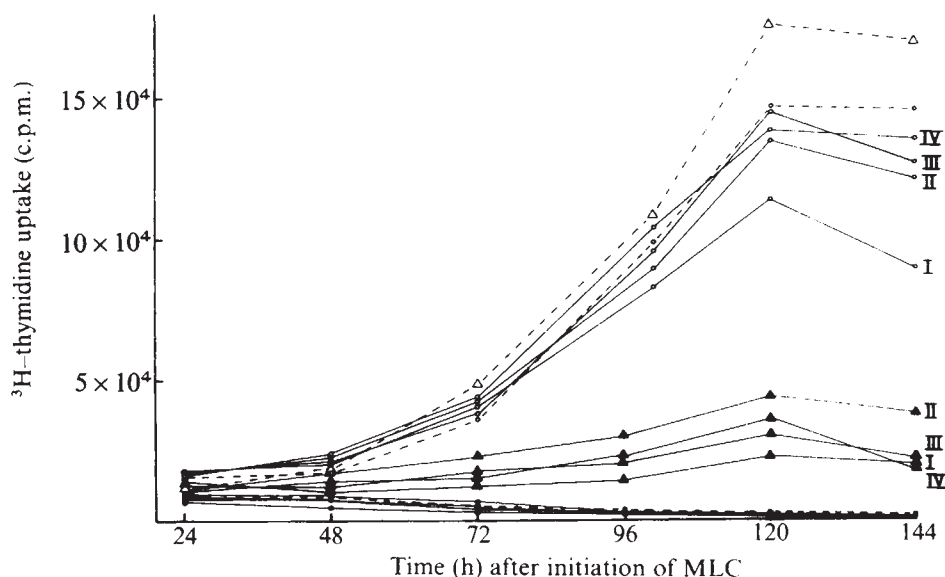
Responder* Stimulator	—	I DBA/2	ACA	—	II DBA/2	ACA	—	III DBA/2	ACA
Day 4	642 ± 58	937 ± 86	1823 ± 71	667 ± 268	1973 ± 93	1724 ± 182	374 ± 103	8065 ± 1961	2488 ± 209
Day 6	302 ± 57	504 ± 143	4517 ± 189	443 ± 66	4429 ± 1163	4964 ± 1225	162 ± 51	28251 ± 2271	12122 ± 580

* I: spleen cells from mice immunised with anti-DBA/2 T blasts at days 0, 60, and 120 and killed at day 130.

II: spleen cells from mice immunised with small, unresponsive CBA/H T cells from the same MLC:s as the I cells.

III: normal CBA/H spleen cells. Culture conditions as described.

Fig. 1 Demonstration of specific unresponsiveness to DA alloantigens induced in Lewis rats immunised three times with 10^7 Lewis-anti-DA T lymphoblasts from day 4 or 5 of primary MLCs. Culture conditions as previously described¹². Individual experimental rats tested day 10 after last immunisation for reactivity against irradiated syngeneic or allogeneic spleen cells. —, Experimental animals; ●, Lewis syngeneic controls; ▲, Lewis against DA; ○, Lewis against; August - - -, Normal Lewis.



adjuvant (DIFCO) and inoculated into one hind leg foot pad. Boosting was carried out using the same number of cells but emulsified in incomplete adjuvant. At indicated time intervals thereafter cells or sera were obtained from the animals and assayed for immune activity.

It could be shown that the present immunisation schedule leads to the induction of specific unresponsiveness against the relevant MHC antigens both in the mouse rat systems. This is depicted in a mouse combination (CBA/H spleen cells from mice inoculated with CBA/H-anti-DBA/2 T blasts displayed specific reduction of unresponsiveness against DBA/a antigens) in Table 1, and a rat combination in Fig. 1 (Lewis spleen cells from Lewis rats immunised with syngeneic blasts reactive against DA cells failed to react against DA but reacted normally against third party August cells). Preliminary kinetic data (not included) have indicated that this selective unresponsiveness may become induced within a few weeks after primary immunisation. That this unresponsiveness of the spleen cells was due to an active process stem from several observations. Using as target cells in a ^{51}Cr release assay CBA/H blasts of different specificity it could be shown that cells from autoimmunised CBA/H mice (immunised with their own anti-DBA/2 blasts) expressed specific cytolytic activity against CBA/H-

anti-DBA/2 blast as depicted in Table 2. If serum from immune animals were present during the incubation, cytotoxicity was still greater in the specific combination. Immune serum could also be shown to make normal CBA/H spleens aggressive against the CBA/H-anti-DBA/2 blasts. These data thus indicate the existence of specific immune reactivity both in cells and sera from autoimmunised mice using as targets the relevant autologous specific T lymphoblasts. The data do not show, however, whether the cytolytic effects observed are entirely due to antibody-dependent cell-mediated cytotoxicity, ADCC, or if specific anti-idiotypic killer T cells are also involved. IgG antibodies are considered to be the most efficient inducers of ADCC¹⁰. ^{125}I -labelled protein A from *Staphylococcus aureus* can be used as a marker of cell-bound IgG antibodies¹¹. Sera from CBA/H mice immunised with CBA/H-anti-DBA/2 T blasts could be shown to react specifically with such blasts and not with other CBA/H blasts, using such ^{125}I -protein A techniques (data not shown). The present data are thus fully compatible with the view that the mice inoculated with their "own" purified T blasts with specificity for a given MHC set of antigens can indeed produce auto-anti-idiotypic antibodies.

Further support for this statement came from the analysis of the reactivity of such anti-lymphoblast sera as to their ability to react with alloantisera containing the relevant antigen-binding, idiotype-positive antibody molecules. It has previously been found possible to detect such anti-idiotypic reactions in the MHC systems using ordinary gel diffusion techniques using repeated applications to the gel basins^{9,12}. Here, we were able to show that the very same serum pool used for the experiments depicted in Table 2 (and also for the ^{125}I -protein A experiments) indeed contained antibodies reactive in a specific way not only with the relevant allo-antibodies (see Table 3) but also with concentrated MLC supernatants of the correct specificity. That the reactive factor in the MLC supernatant did indeed react with the relevant H-2 antigens was shown by specific absorptions to the relevant tissues.

In summary, we feel confident to make the following conclusions. It is possible to induce a state of selective unresponsiveness or hyporeactivity against a specific set of MHC antigens using autoimmunisation with the individual's own, *in vitro* educated T blasts with specificity for these antigens. We also know that at least in some individuals these immunisation procedures will lead to the production of detectable amounts of autoanti-idiotypic antibodies with specificity for relevant antigen-binding receptors. As previous experiments using purified soluble idiotype

Table 2 Demonstration of specific lytic activity of spleen cells and serum from autoimmunised CBA/H mice

Effector cells	Serum†	Target blasts, % kad	^{51}Cr release‡ kaf	^{51}Cr release‡ k ConA
I	5% FCS	51	16	< 0
I	2% NMS - 0.1% IS	30	< 0	< 0
I	2% NMS	18	< 0	1
II	5% FCS	< 0	0	< 0
II	2% NMS + 0.1% IS	47	11	6
II	2% NMS	< 0	0	< 0
III	5% FCS	< 0	< 0	< 0
III	2% NMS + 0.1% IS	24	< 0	< 0
III	2% NMS	5	< 0	< 0

* Effector cells. I: spleen cells from CBA/H mice immunised three times with 10^7 CBA/H-anti-DBA/2 T MLC blasts, last time 10 d before testing II: spleen cells from CBA/H mice immunised three times with CBA/H small T lymphocytes from the same MLCs as the anti-DBA/2 T blasts; III: spleen cells from normal CBA/H mice.

† FCS: foetal calf serum; NMS: normal CBA/H serum; IS: serum from the CBA/H mice immunised with CBA/H-anti-DBA/2 T blasts. All sera 56°C 20 min inactivated before use.

‡ ^{51}Cr -labelled T blasts of CBA-anti-DBA/2 (kad), CBA/H-anti-ACA (kaf) and CBA/H-ConA blast were used as target in a cytotoxic release assay run at 6 h at a 100:1 ratio effector/target cells.

Table 3 Serum from CBA mice immunised with CBA-anti-DBA/2 T blasts contain anti-CBA-anti-DBA/2 idiotype antibodies as detected in gel diffusion experiments

Absorbed with	Tested against:	Positive line
—	CBA normal serum	—
—	CBA-anti-DBA/2 serum	+
—	CBA-anti-DBA/2 MLC sup.*	+
—	Foetal calf serum	—
—	CBA-anti-DBA/2 MLC sup., A	—
—	B	—
—	C	+
—	D	+
—	E	—
—	CBA-anti-DBA/2 MLC sup.	—
—	abs with DBA/2 spleen	—
—	abs with CBA spleen	+
—	abs with ACA spleen	+
DBA/2 spleen cells	CBA-anti-DBA/2 MLC sup.	+
ACA spleen cells	CBA-anti-DBA/2 MLC sup.	+
CBA spleen cells	CBA-anti-DBA/2 MLC sup.	—

*MLC sup: supernatant from day 5 MLC of CBA-anti-DBA/2. Supernatant concentrated 100 times before testing in gel diffusion. Two different batches positive. Supernatant fractions A-E: mean fractions after Sephadex G-200 fractionation. C and D: fractions in the size range of human serum albumin or slightly above, B: size range of IgG, A: size range of IgM.

antibodies as immunogen in the present system could be shown to lead to selective unresponsiveness to the relevant histocompatibility antigens¹ we deem it most likely that the present unresponsiveness as measured in the MLC tests is induced through the same basic mechanism, namely that of autoanti-idiotypic immunity. Thus, the present experimental approach would seem to promise to provide a general tool to enrich *in vitro* for relevant antigen-binding, idiotype cells using the latter to induce productive auto-immune reactions leading to specific unresponsiveness to the relevant antigens. We are presently trying to study how this approach can be used in the induction of specific transplantation tolerance as well as in attempts to break existing states of delayed hypersensitivity.

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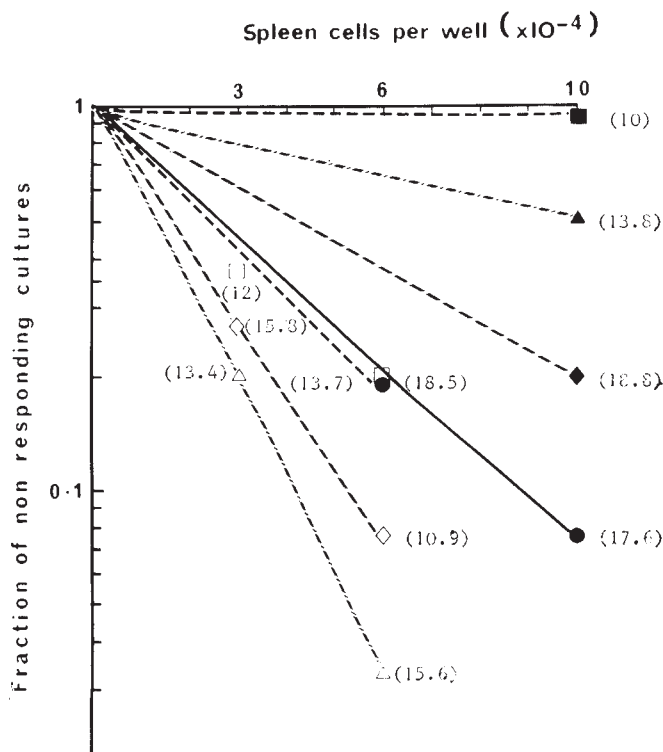
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Evidence for the inactivation of precursor B cells in high dose unresponsiveness

THE mechanisms of tolerance induction, a state of specific antigen induced unresponsiveness in lymphocytes, are still ill understood. The impetus to generate information on these mechanisms derives from the practical importance of understanding "self-tolerance". Both T (thymus-derived)

and B (bone marrow-derived) lymphocytes can be rendered specifically unresponsive to antigen. This paper examines the nature of the unresponsiveness induced in B cells by high doses of two types of antigen; firstly by a set of hapten conjugates known to be thymus-independent antigens (DNP-SIII, DNP-Levan, DNP-Dextran)¹ and secondly by a known thymus-dependent conjugate (TNP-BSA). It is now well authenticated that for efficient antibody production B cells need to proliferate after which members of the proliferated clone may differentiate into antibody producers. It is therefore important to establish whether unresponsiveness induced in B cells occurs by inactivation of the precursor cell or by an effect on the derived clone. We here demonstrate that *in vitro*, B cell unresponsiveness induced by both kinds of antigens (thymus-dependent and independent) results solely from the inactivation of the precursor hapten specific B cells, so that these cells cannot expand into

Fig. 1 The effect of 48 h of preculture of spleen cells with DNP-polysaccharide conjugates on the frequency of precursors responding to 0.1 $\mu\text{g ml}^{-1}$ TNP-LPS. 1×10^7 spleen cells ml^{-1} from normal CBA mice were incubated with various conjugates in 50 mm tissue culture dishes (Falcon) in 5-ml volumes. After 48 h spleen cells were washed and spun through heat inactivated foetal calf serum. Varying numbers of such cells were supplemented with irradiated (1,200 rads from a ^{60}Co source) spleen cells and cultured for 3 d in micro-titer plates in RPMI containing 5% foetal calf serum and additives³. All cultures were challenged with 0.1 $\mu\text{g ml}^{-1}$ TNP-LPS. Positive wells were then determined by PFC assay of the micro-cultures. In brief the culture tray was flooded with 10 ml Eagles HEPES medium, and washed. Each well was sampled and anti TNP-PFC were enumerated by a modification of the Jerne PFC assay using hapten-conjugated donkey erythrocytes (TNP-DRBC). Results are expressed as the fraction of non-responding wells for each cell input. Average clone sizes are displayed in the parentheses. Average clone sizes were estimated by first establishing the frequency of hapten reactive spleen cells using the Poisson formula⁴. From this value the total number of hapten-specific precursors (N) distributed into 30 wells can be estimated. Where ΣPFC is the total number of PFC found in 30 wells $\Sigma (\text{PFC}/N)$ is the average number of PFC per hapten-specific B cell. Average clone sizes are displayed in parentheses. ●; Nil; ■, 10 $\mu\text{g ml}^{-1}$ DNP₈SIII; □, 0.1 $\mu\text{g ml}^{-1}$ DNP₈SIII; ◆, 10 $\mu\text{g ml}^{-1}$ DNP_{2.5}Levan; ◇, 0.1 $\mu\text{g ml}^{-1}$ DNP_{2.5}Levan; ▲, 0.3 $\mu\text{g ml}^{-1}$ DNP_{2.8}-De; ▽, 0.03 $\mu\text{g ml}^{-1}$ DNP_{2.5}-De.



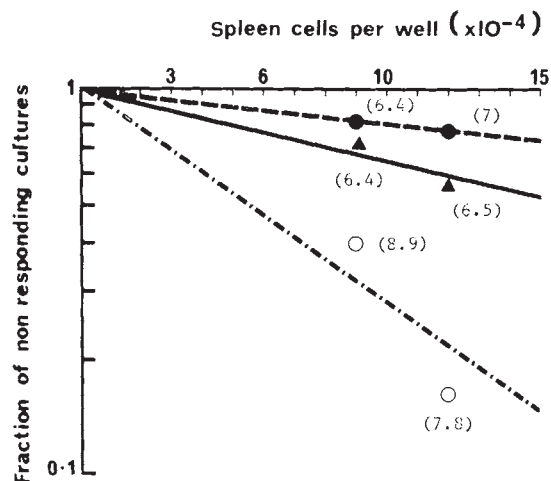


Fig. 2 The effect of 48 h of preincubation of spleen B cells with TNP₁₀BSA on the frequency of precursors responding to TNP-LPS and their secreting clone size. Spleen cells from adult thymectomised, lethally irradiated, bone marrow repopulated CBA mice were incubated for 48 h at 1×10^7 cells ml⁻¹ as before with no TNP₁₀BSA (○), $50 \mu\text{g ml}^{-1}$ TNP₁₀BSA (▲), or $500 \mu\text{g ml}^{-1}$ TNP₁₀BSA (●). After this the cells were washed and redistributed as in Fig. 1. All wells were supplemented with irradiated AT×Bm cells to a final cell concentration of 1.5×10^5 per well. 30 wells were established for each treatment group. All microcultures were then challenged with $0.1 \mu\text{g ml}^{-1}$ TNP-LPS and anti-TNP responding cultures enumerated as before. Each point represents the mean of 30 microcultures. The conjugation ratio of TNP₁₀BSA is expressed as moles of hapten per mole of BSA.

normal antibody producing clones, rather than by any mechanism which may have affected the clone size such precursor cells could yield.

Spleen cells from CBA mice were passed through Sephadex G10 columns² to eliminate 'background' antibody producing cells (PFC). These cells were incubated with various concentrations of the hapten polysaccharide conjugates in bulk cultures (1×10^7 ml⁻¹ in 5-ml volumes) for 48 h and were then washed, established in microcultures in $10 \mu\text{l}$ volumes in microtest 3034 plates (Falcon)³, and re-challenged with optimal concentrations of Trinitrophenylated-lipopolysaccharide (TNP-LPS), another thymus-independent antigen. By titrating various numbers of such pretreated spleen cells into a filler population of irradiated cells it was possible to examine the frequency of the responding B cell using Poisson distribution analysis⁴. With the resultant knowledge of precursor frequency, and having calculated the total number of PFC a given number of spleen cells could produce, we were able to make estimates of average clone size.

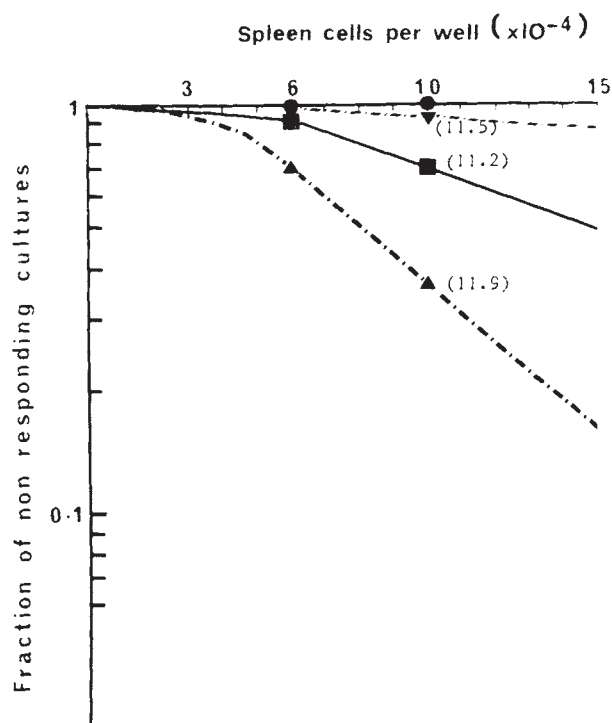
Figure 1 shows that cells precultured for 48 h in the presence of supraoptimal amounts of DNP-Dextran, DNP-Levan or DNP-SIII exhibited lower frequencies of cells reactive to TNP-LPS than controls. It should be pointed out that the pattern of responses observed with these three conjugates correlates well with their immunogenic and tolerogenic properties *in vivo*⁵ and in *in vitro* Marbrook cultures¹. Estimates of the average clone size of all pretreated groups responding to TNP-LPS showed that the number of PFC per anti TNP B cell was constant at approximately 10-19 PFC per precursor cell. It can be seen from Fig. 1 that the small variations in clone size estimates did not correlate with the tolerogenicity of the conjugates. This suggests that the reduced responses induced by high concentrations of T-independent antigens is primarily a consequence of inactivation of precursors rather than an influence on their subsequent clonal progeny.

An analogous situation was also observed after exposure of spleen cells from adult thymectomised irradiated and bone marrow repopulated CBA mice to high concentrations

of TNP₁₀BSA, a relatively oligovalent ligand. After a preincubation period of 48 h, free antigen was washed away, and frequencies and clone sizes of hapten-reactive B cells were determined on rechallenge with TNP-LPS. Preincubation of cells with $50 \mu\text{g ml}^{-1}$ TNP₁₀BSA produced more than a threefold reduction of hapten specific precursor cells reactive to TNP-LPS, whereas $500 \mu\text{g ml}^{-1}$ TNP₁₀BSA produced more than a sixfold reduction (Fig. 2). Estimates of the average clone size (PFC/precursor) are also shown in the inserts for each point in the figure, and were constant at approximately 6-8 PFC per B cell.

Similarly, if one examines the frequency of cells reactive to DNP_{2,8}Dextran (DE) in a normal spleen population using the microculture system, it is clear that at supraoptimal doses of antigen a diminished frequency of reactive cells is seen but clone sizes stay constant. Thus, in Fig. 3 is shown the data from an experiment where different concentrations of DNP-DE were added to groups of 30 microcultures, each group containing varying numbers of normal cells maintained at a constant cell concentration with the irradiated filler cells. After 4 d of culture both the number of wells giving a positive anti-DNP response and the total number of anti-DNP PFC generated for each group were determined. The frequency of reactive cells was approximately 1×10^{-5} with $0.03 \mu\text{g ml}^{-1}$ of DNP-DE but increasing concentrations resulted in lower frequencies of responding cultures, such that at $1 \mu\text{g ml}^{-1}$ no responding wells were detected (Fig. 3). Calculations of average clone size (that is

Fig. 3 The effect of various doses of DNP_{2,8}De (B1299) on the frequency of anti-TNP precursor B cells, and on the clone size such cells can generate. Sephadex G10 treated spleen cells from normal CBA mice were incubated in microcultures at various numbers in a final cell concentration of 10^7 ml⁻¹ maintained by the addition of irradiated filler cells. Cultures were challenged with various concentrations of DNP-De. After 4 d of incubation wells were harvested and assessed for PFC as before. Results are expressed as the fraction of non-responding wells for each cell input. Each point represents the fraction of non-responding cultures from 30 wells sampled. Average clone sizes are displayed in parentheses in the figure. ▲, $0.03 \mu\text{g ml}^{-1}$ DNP-De; ■, $0.1 \mu\text{g ml}^{-1}$ DNP-De; ▼, $0.3 \mu\text{g ml}^{-1}$ DNP-De; ●, $1.0 \mu\text{g ml}^{-1}$ DNP-De. DNP-De was prepared as described previously and the conjugation ratio is expressed for molecular weight of 40,000.



PFC per B cell) were found to be relatively similar at all antigen concentrations where positive wells were detected (approximately 11 PFC/B cell). These values for clone sizes are again stated by each point in the figure. The data suggest that supraoptimal concentrations of this thymus-independent conjugate produced their inhibition of the anti-DNP response by inhibiting the precursor B cell without influencing the clones derived from those which escaped inhibition. Identical results have also been obtained with varying concentrations of DNP_{2,5} levan.

These results obtained in the defined conditions of the microcultures lead us to conclude that the interaction of B cell surface receptors with high concentrations of multivalent thymus-independent antigens results in a situation where the cell either enters into a division and differentiation 'programme' to generate a burst size constant for that antigen, or does not begin the cycle at all. It is clear that such inhibited cells are refractory to restimulation at least in the short term *in vitro* situation. B-cell unresponsiveness generated by the T-dependent ligand TNP-BSA is similar in this respect. It remains to be determined whether these refractory states of the precursor B cells are permanent or reversible.

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Lack of suppressive effect of α -foetoprotein on development of experimental allergic encephalomyelitis in rats

α -FOETOPROTEIN (AFP) is a major glycoprotein that occurs in substantial amounts in mammalian sera and body fluids during foetal and early neonatal life. Unusually high levels of AFP have been observed in the amniotic fluid in association with congenital abnormalities of the central nervous system (CNS)^{1,2}. Abnormal amounts have also been found in adults with hepatic and gastrointestinal malignancy^{3,4}. AFP has attracted attention as a potential immunoregulatory protein with suppressive activity for immune responses of mammalian lymphoid cells⁵⁻⁷, including auto-sensitisation. For example, normal rat serum prevents adult rat lymphoid cells from reacting immunologically to self-antigens *in vitro*⁸. In rats with experimental allergic encephalomyelitis (EAE), a prototypic autoimmune disease of the CNS⁹, a serum factor has been reported which causes suppression of proliferative responses of rat lymphoid cells exposed to mitogens¹⁰. Peak serum suppressive activity paralleled increasing α -globulin concentration and coincided with reversal of disease. We have reported¹¹ that development of EAE is impaired in suckling rats of an age when high levels of AFP would be expected. Using two rat model systems, we have dissociated

AFP production, assessed by serum concentration, from the occurrence of EAE. Our findings suggest that AFP does not have a major suppressive role in the development of this autoimmune disease.

We took advantage of two available model systems which utilise rats sensitised to nervous tissue-adjuvant emulsions. The first involves adult Buffalo strain rats bearing designated Morris hepatomas which produce diverse amounts of AFP¹², and the second involves the diminished occurrence of EAE in suckling Lewis rats 4-14 d old¹¹.

To prepare the first model, adult female Buffalo rats, 8-12 weeks old, except in one experiment with 30-week-old animals, were implanted with cells from tumours that produced either high levels of AFP (Morris hepatoma 7777) or low levels of AFP (Morris hepatoma 5123)¹². Palpable tumours were usually evident within 2 or 3 weeks, at which time the tumour-bearing rats and uninoculated controls (of identical age and received in the same shipment of animals used for implantation of tumour cells) were sensitised to guinea pig spinal cord (GPSC) in complete Freund's adjuvant (CFA) as described before¹³.

To prepare the second model, suckling Lewis rats, an isohistogenic strain known to be not only highly susceptible to EAE¹⁴ but, from observations in our laboratory, far more susceptible than Buffalo rats, were similarly sensitised with GPSC-CFA at different times after birth¹¹.

All rats were checked daily for clinical neurological signs—limp tail, ataxia and hindleg weakness or paralysis. Rats were killed and CNS tissue was collected and processed as follows. Formalin-fixed blocks of cerebrum, mesencephalon, cerebellum-pons and spinal cord (representing cervical, thoracic-lumbar and cauda-equina regions) were embedded in paraffin, sectioned at 5-7 μ m, stained with haematoxylin and eosin and examined microscopically for evidence of perivascular mononuclear cellular infiltrates characteristic of EAE. AFP levels in sera collected from normal or sensitised rats at appropriate times were measured by single radial diffusion in agar¹⁵.

In six experiments summarised in Table 1, Buffalo rats bearing Morris hepatomas had a lower incidence of both clinical signs and cellular infiltrates indicative of EAE compared with controls. This was true irrespective of which type of tumour had been inoculated. Neither the incidence nor the severity of EAE in individual rats could be related to the serum levels of AFP at the time of death. Moreover, there was no relationship in individual rats between incidence of EAE and the size of tumours (based on wet weight of masses excised at autopsy), or between tumour size and AFP serum levels at the time of death. From these data it was possible to conclude that AFP does not directly suppress the occurrence of EAE, and the diminished incidence of the disease is probably a result of the tumour *per se*.

As anticipated from somewhat higher AFP levels in 3-week-old compared with adult rats¹⁶, we found that neonatal Lewis rats had very high levels of serum AFP (Fig. 1), in

Table 1 Occurrence of EAE in Buffalo rats bearing Morris hepatomas

Type of tumour injected	Occurrence of EAE		AFP levels (μ g ml ⁻¹)*	
	Clinical signs	Cellular infiltrates	Range	Average
None	18/22	21/22	< 20	< 20
Morris 7777	4/11	6/11	1,320-4,130	2,194
Morris 5123	0/5	3/5	110-258	170

Rats were injected intracutaneously in six sites over the upper back and one site on the ventral neck (0.1 ml per site) with a 33% GPSC tissue homogenate in 0.25% phenol H₂O and emulsified with an equal volume of CFA (BCG 4 mg ml⁻¹).

*AFP levels at death, 19-24 d after sensitisation.

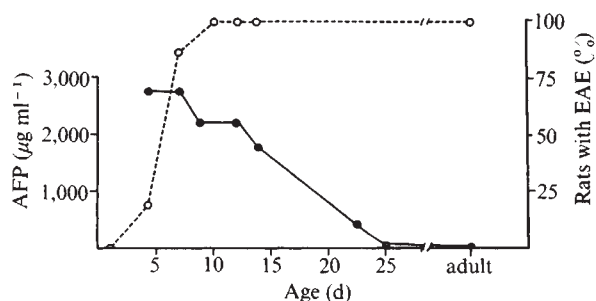


Fig. 1 AFP levels (●) and occurrence of EAE (○) in rats as a function of age. Suckling and adult Lewis rats were sensitised as described in Table 1, except that injection was into four sites over the upper back and one site on the ventral neck (0.05 ml per site) by the subcutaneous or intracutaneous routes.

the range of $2,750 \mu\text{g ml}^{-1}$ at 4 and 7 d of age and then gradually declining towards adult values after 25 d of age. Figure 1 also shows that 7-d-old or older suckling rats sensitised with GPSC-CFA at various ages were susceptible to EAE, although they had levels of AFP comparable with those of tumour-bearing rats (Table 1). There was no significant difference in the latent period for development of EAE (the time between sensitisation and the onset of clinical signs) in these suckling rats compared with adult rats. (A lengthening of the latent period in suckling rats might have represented a subtle manifestation of an AFP-mediated suppressive effect on development of disease, at least until AFP levels decline with increasing maturation (Fig. 1).) The fact that 7-d-old rats can develop EAE even with AFP levels comparable with or identical to those of 4-d-old animals, which have a low incidence of EAE, is our most conclusive evidence that AFP does not explain the lack of disease in 4-d-old rats.

AFP concentrations considerably greater than normal occur in association with congenital malformations of the CNS^{1,2} and ataxia telangiectasia, an immunodeficiency disease involving the CNS³. We therefore investigated whether the injury to the brain and spinal cord which is characteristic of EAE is accompanied by increased concentrations of AFP in serum. Twenty-four adult 8–12-week-old Lewis and Buffalo rats were bled 12–24 d after being sensitised to GPSC-CFA, a time when inflammation occurs in the CNS in association with the appearance of clinical neurological signs of EAE. Levels of AFP were not appreciably different from those of adult rats before sensitisation (Fig. 1). Thus, injury to the CNS does not result in markedly increased AFP levels as occurs with ataxia telangiectasia and malformations of the CNS. Furthermore, AFP does not increase appreciably as part of the "acute phase reaction" accompanying inflammation irrespective of its nature, which is commonly observed with serum proteins, such as fibrinogen and complement.

The incidence of EAE in rats bearing Morris hepatomas was less than in simultaneously sensitised adult Buffalo rats bearing no tumours (Table 1). But disease was not suppressed in non-tumour-bearing suckling Lewis rats with comparable levels of AFP (Fig. 1). We suspect that the diminished incidence of disease associated with the Morris hepatoma reflects other tumour-related factors, such as diminished host inflammatory and/or immune responses which are known to be decreased in the presence of a critical burden of tumour cells¹⁸. AFP has been reported to have quite a variable suppressive effect on *in vitro* lymphocyte proliferation induced by different mitogens¹⁹. Other workers^{3–7} have suggested that AFP has an unequivocal suppressive effect *in vitro* on antibody production as well as on lymphoid cell proliferation induced by either allogeneic cells or mitogens. Studies *in vivo*, on the other hand, have demonstrated preferential suppression of IgA compared with IgG antibody responses to sheep red blood cells²⁰.

These various results suggest that the net suppressive effect of AFP in a given situation depends on the type of antigenic stimulus and the intensity of the resulting immune response. Vigorous antigenic stimulation with spinal cord and Freund's adjuvant as used to induce EAE, could conceivably bypass a potential suppressive effect of AFP in rats. In this regard it may be useful to determine the effect of AFP on other autoimmune diseases induced by different types of antigenic stimulation, such as thyroiditis, orchitis and adrenalitis.

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Murine neuroblastoma cured *in vivo* by an antibody-dependent cellular cytotoxicity reaction

CYTOLYSIS of antibody-coated tumour cells mediated by reticuloendothelial (RE) or bone marrow cells is a phenomenon of unknown biological relevance^{1–4}. The specificity of the reaction lies in the antibody^{5,6}; probably most or all cells able to recognise the Fc fragment of the antibody molecule can mediate this reaction, including lymphocytes and monocytes^{6,7}, circulating cultured transformed lymphoblasts⁸, polymorphonuclear leukocytes⁹ and malignant reticulum cells¹⁰. In tumour systems the contribution of this reaction is difficult to distinguish *in vivo* from other cytolytic phenomena, including complement-dependent antibody-mediated cytotoxicity, T-cell cytotoxicity or cell killing exerted by "armed" macrophages^{11,12}, all of which may be active in various degrees in different experimental circumstances. But antibody-dependent cellular cytotoxicity (ADCC), as this phenomenon has been termed¹³, seems to be of potential therapeutic interest because both its magnitude and specificity depend on specific antibody levels, and antibody levels may be potentially more manipulable in man than are the cellular elements of a host anti-tumour response. In addition, there is preliminary evidence that

Table 1 *In vitro* ADCC against MNB cells

Sample	c.p.m.	±s.e.	⁵¹ Cr % Specific release
MNB cells, control	1140	38	0
MNB cells+RSC	1055	81	0
MNB cells, freeze-thawed	2019	55	100
MNB cells+AS 1:50+RSC	1528	34	44
MNB cells+AS 1:250+RSC	1570	44	49
MNB cells+AS 1:6250+RSC	1159	36	2
MNB cells+AS 1:31250+RSC	1088	26	0

Log phase MNB cells were collected by treatment with 0.25% Viokase (Grand Island Biological) for 2–5 min at room temperature. After aspiration and washing once in full growth medium, cells were resuspended in DMM to a final concentration of $2-4 \times 10^6 \text{ ml}^{-1}$. They were then labelled with 300–400 μCi of sodium ⁵¹Cr-chromate (New England Nuclear Corp.) by incubating in tissue culture conditions in an incubator perfused with water-saturated 95% air: 5% CO₂ at 37 °C for 30 min with intermittent shaking every 10 min. The cells were then washed three times by centrifugation and resuspension in Hanks solution followed by final resuspension at $2 \times 10^4 \text{ cells ml}^{-1}$ in DMM. Heat-inactivated (56 °C for 30 min) rabbit anti-MNB serum was then added to 1.0 ml of cells to give the final concentrations indicated. Each sample was then incubated at 37 °C for 30 min with shaking every 10 min. RSC were isolated by gentle mincing of a fresh spleen from an adult Wistar rat. RSC suspended in DMM ($2 \times 10^7 \text{ ml}^{-1}$) were then added (0.1 ml) to the antibody-coated ⁵¹Cr-labelled MNB cells to give a final ratio of 100:1 RSC:MNB cells. The mixture was then incubated for about 18 h at 37 °C in the tissue culture conditions described above. Each sample was then centrifuged for 10 min at 118g. A sample of the supernatant (0.5 ml) was then aspirated carefully from above the cell pellet and counted in a Beckman gamma scintillation counter. Duplicate MNB cell samples processed in an identical way without anti-MNB serum or RSC treatment were used to determine spontaneous ⁵¹Cr release. "Total" release was measured by freezing and thawing similar duplicate untreated samples three times. Non-immune serum was without effect.

%Specific release =

$$\frac{\text{c.p.m. test sample} - \text{c.p.m. control (MNB cells alone)}}{\text{c.p.m. freeze/thaw} - \text{c.p.m. control MNB alone}} \times 100$$

fixed tissue macrophages may be active in eliminating circulating metastatic cells by this mechanism¹⁴. It was therefore interesting to determine whether functional ADCC could be demonstrated *in vivo* independent of other cytolytic mechanisms since its demonstration has so far been limited to *in vitro* assays.

We used the C-1300 murine neuroblastoma (MNB) line carried in syngeneic A/J mice. Spindle-cell sarcoma I (SaI) was used as a control; SaI is also A-strain-derived and has a 100% transmission rate in syngeneic A/J mice. The MNB system is of special interest since C-1300 MNB cells remain capable of neuronal differentiation when passage *in vivo* many times^{15,17}. The disease closely resembles human neuroblastoma (HNB) both in this respect and in its response to chemotherapy¹⁸ and radiotherapy¹⁹. HNB is the most common solid tumour of early childhood with a peak incidence in the second year of life²⁰. It is characterised by a fulminant proliferation of immature sympathetic neuro-

blasts. Differentiated MNB cells have been shown to develop antigenic changes similar to those occurring during normal neuronal development^{21,22}. Most importantly, HNB patients and their mothers have circulating antibodies and cytotoxic lymphoid cells able to recognise HNB cells *in vitro*²³. Thus HNB patients potentially possess the active components for ADCC and augmentation of such a response might have therapeutic value. The experiments reported here indicate that ADCC with significant specificity can be demonstrated against MNB cells both *in vitro* and *in vivo* at levels which are therapeutically curative.

To obtain antisera against MNB cells adult female New Zealand rabbits were immunised by four injections of 10^7 log phase MNB cells grown in Dulbecco's modified medium (DMM) *in vitro* as described before²⁴. Ten days after the fourth injection, the animals were bled and titred for anti-MNB activity. A single lot of antiserum was used for all the experiments. ADCC was first evaluated *in vitro* using a ⁵¹Cr-release method²⁵ outlined in Table 1. Neither antibody alone nor effector RE cells (rat spleen cells, RSC) were active alone while MNB cells coated with antibody and then exposed to RSC showed significant cell killing. The results indicated that ADCC was demonstrable in the system.

To determine whether ADCC could occur *in vivo*, MNB were first coated with antibody *in vitro* and cells were then injected subcutaneously into the right flank of syngeneic A/J mice. The ability of RSC cells to induce ADCC was then evaluated by a second injection of RSC in the general location of the original MNB injection. In each case separate needles, syringes and injection points were used. The animals were then watched for the time of appearance of palpable tumour (>1.0 mm) and subsequent survival. The pooled results (Table 2) of three separate experiments showed that neither *in vitro* antibody treatment nor RSC alone were effective. Antibody alone had essentially no effect ($P=0.62$), while RSC injected against non-antibody coated MNB cells delayed the onset of palpable tumour slightly with statistically significant lengthening of the median survival ($P=0.003$). The degree of protection was a function of the ratio of RSC to MNB cells injected. When antibody-coated MNB cells were studied, low ratios of RSC:MNB cells (10:1) led to a statistically significant additional increment in the delay of onset and median survival ($P<0.001$) while higher ratios (50:1) further extended the time of onset and survival of those animals which developed tumours. In addition, one out of every three animals treated at the 50:1 ratio of RSC:MNB cells failed to develop tumours. Because fewer than 10 MNB cells can induce lethal tumours in A/J mice²⁶ the results suggest that ADCC is highly efficient, having achieved about a 5 decade kill (which means that 1 in 10^5 survive) in about one-third of the mice. This phenomenon was dependent on injection of the effector RSC in the general area of the original MNB

Table 2 *In vivo* ADCC against MNB cells

Sample	Median onset (d)	s.e.m. (d)	Median survival (d)	s.e.m. (d)	Cures
MNB alone	9.93	0.35	25.6	0.86	0/15
MNB+AS	10.53	0.42	26.2	0.84	0/15
MNB+RSC (10:1)	13.78	0.36	31.1	1.27	0/9
MNB+RSC (50:1)	14.3	0.40	32.4	1.25	0/10
MNB+AS+RSC (10:1)	16.13	0.78	33.5	1.44	0/15
MNB+AS+RSC (50:1)	21.54	1.77	37.6	1.71	5/15
MNB+AS+RSC (50:1) ectopic	11.8	0.73	28.4	2.29	0/5

Eight-week-old A/J mice were inoculated subcutaneously in the right flank with 10^6 MNB cells in 0.1 ml which had been either pretreated (0.1 ml AS or dilution per 10^6 cells for 60 min at 37 °C) with rabbit anti-MNB serum (AS) or not (MNB alone). The inoculating needle was withdrawn and in treated animals a second needle was inserted in the same general area and either 0.1 or 0.5 ml of RSC ($10^6 \text{ cells ml}^{-1}$) was injected to give the indicated ratios of RSC:MNB cells. In one series of experiments, the RSC cells were injected in the left flank (ectopic injection). The mice were then watched for the time of appearance of palpable tumour and for survival. Cures indicate those animals failing to develop tumours within 60 d. In the series in which "cures" were obtained only animals developing tumours were included when median survival was calculated. The paired *t* test was used to calculate statistical significance between each group and MNB controls.

Table 3 Specificity of *in vitro* ADCC against MNB cells

Sample	Median onset (d)	s.e.m. (d)	Median survival (d)	s.e.m. (d)
SaI alone	6.0	0.0	17.4	0.87
SaI + AS (ab) + RSC	6.2	0.20	19.0	1.58
MNB alone	11.8	0.73	31.2	1.32
MNB + AS (ab) + RSC	22.2	2.03	48.8	2.92

A/J mice were injected with either uncoated SaI or MNB cells as described in Table 2. Another set of mice received similar inoculations after coating with anti-MNB serum which had previously been repeatedly absorbed with SaI cells (10^7 per ml of anti-MNB serum) until complement-dependent antibody mediated cytotoxicity was not demonstrable by Trypan blue exclusion. The animals receiving AS-coated cells subsequently received an inoculation of RSC (50:1) at nearby site, as described in Table 2.

injection. Ectopic injection into the left flank of recipient mice was essentially without effect (Table 2).

To check the specificity of the reaction, the anti-MNB serum was absorbed exhaustively with SaI cells as described in Table 3. Absorbed antisera had no effect on either the time of tumour appearance or the time of death of mice receiving SaI cells and high levels of RSC. The antisera, however, were still effective in delaying the time of appearance of MNB tumours ($P < 0.01$) and the time of death of the animals ($P < 0.001$).

These experiments suggest that the MNB system is interesting in the overall approach to HNB treatment and also as a general approach to the study of *in vivo* ADCC phenomenon as a potential means of cancer therapy. The data indicate clearly that ADCC does not occur only *in vitro* but also *in vivo*. But to be effective a relatively high ratio of effector cells is required and these must be relatively near the target cells. Ectopic exposures of even favourable effector cell ratios were ineffective (Table 2). But with appropriate antisera and physical circumstances, the effect is specific and very striking. In optimum conditions the extent of tumour cell killing greatly exceeds that attainable by aggressive chemotherapy¹⁸ and has no detectable deleterious effect on the "cured" mice. It is very interesting that a small amount of xenogeneic antiserum that is itself ineffective (that is not rendered cytotoxic by *in vivo* exposure to mouse complement) can be curative in the ADCC system. *In vitro* activation of armed macrophages was reported by Van Loveren and Den Otter. Although the nature of the activating substance was not identified it also proved therapeutically effective in rendering normal macrophages tumoricidal²⁷.

We suggest that *in vitro/in vivo* modes for evaluating ADCC may be useful in the evaluation of multidisciplinary cancer therapy. For example, it is well established that MNB is highly resistant to *in vivo* chemotherapy, as is its human counterpart¹⁸. Multimodality treatment regimes can therefore be evaluated by this type of model. We have shown that liver parenchymal RE elements (presumably Kupffer cells) can effect moderate levels of tumour cell killing by means similar to or identical with ADCC¹⁴. The results shown here indicate that tumour cell killing *in vivo* by ADCC mechanisms can be active in tissues but is subject to significant anatomical limitations. We propose that human tumours with favourable anatomical sites (such as ovarian carcinoma) may benefit from this approach. The *in vitro/in vivo* model such as exemplified here should facilitate evaluation of specific tissue limitations and perhaps the study of means of stimulating RE cell migration in abrogating such limitations. Unlike T-cell-mediated immunity, the specificity of ADCC is antibody-mediated. Species barriers may therefore be less of a problem during passive immunotherapy because xenogeneic antiserum is clearly effective. Moreover, passive transfer of immunity ("arming") by antigen-antibody complexes is a characteristic of this type of immunity^{11,28}. These features suggest that the ADCC reaction may prove manipulable in human

tumour immunotherapy and warrants further exploration in this context.

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Serological reactions against glycolipid-sensitised liposomes in multiple sclerosis

MULTIPLE SCLEROSIS (MS) is a neurological disease of unknown aetiology which involves the destruction of central nervous system myelin¹. Although recent results have implicated a possible viral agent^{2,3}, the search continues to identify an antigen which might provoke a postulated autoimmune reaction⁴. Such a role has been suggested not only for proteins, but recently also for myelin glycolipids, particularly galactocerebroside^{5,6}. However, to demonstrate immunological reactions against lipid antigens presents special problems: solubility, accessibility of reactive groups,

the anticomplementarity of many lipids and the requirement for auxiliary lipids, lecithin and cholesterol⁷. We have used a model membrane system which obviates these difficulties to survey sera from MS patients for reactivity against crude extracts of white matter lipids as well as various glycolipids. This test demonstrated antibodies reacting against cerebroside, gangliosides and white matter lipids in some of the MS sera tested, but reactions against digalactosyl diglyceride (an α -galactoside) were more frequent, and were found more often in MS patients than in normal controls, or those with other neurological diseases.

The assay employing liposomes (vesicles consisting of concentric lipid bilayers entrapping between them an aqueous compartment) is based on the fact that these model membranes can be lysed, in the presence of complement, by appropriate antisera against lipid haptens in their membranes⁸. The system seemed promising as a tool in the search for anti-lipid antibodies in MS sera, because it has been used to investigate reactions against several galactolipids in immunised rabbits⁹ and to identify antibodies to lipids of *Schistosoma* parasites¹⁰.

Multilamellar, unsonicated liposomes containing distearoyl lecithin, cholesterol and dicetyl phosphate in a molar ratio of 2:1.5:0.2, sensitised as described⁹, and incorporating umbelliferyl phosphate as a marker¹¹, were prepared. Lysis was measured by the inclusion, in the external medium, of alkaline phosphatase which converts released marker to fluorescent umbelliferone. The method of Six *et al.*¹¹ was scaled down ten-fold to obtain greater sensitivity (the preparation of unilamellar liposomes was not found necessary). Results were expressed as percentage of total marker released. Total entrapped marker was determined each day by lysing 1 μ l of liposomes in acetone. Controls without serum, or containing heated complement, were analysed in parallel, as well as unsensitised liposomes. (Any lysis obtained with these was subtracted.) Liposomes made with dipalmitoyl lecithin gave similar results to those containing distearoyl lecithin.

Specific antisera to the lipids used were raised in rabbits⁹ to determine the sensitivity of each batch of liposomes. Cross-reactivity experiments with these were in accord with published findings⁹; anticerebroside sera cross reacted to some extent with liposomes containing digalactosyl diglyceride (DGD), but anti-DGD sera did not lyse liposomes containing cerebroside or gangliosides. Sera from rabbits immunised with normal human white matter (NHWM)

lysed liposomes sensitised with galactocerebroside but not glucocerebroside, confirming results obtained by other methods¹².

Human sera were obtained through the services of the University of California at Los Angeles MS clinic. They were heated (56 °C, 30 min), divided into small aliquots and stored at -20 °C.

Initial experiments with MS sera showed that: (1) sera which lysed NHWM liposomes also reacted with cerebroside and gangliosides; (2) galactocerebroside liposomes were not lysed preferentially (only eight out of 23 patients tested gave positive reactions); (3) liposomes sensitised with digalactosyl diglyceride (DGD), however, were lysed by some sera which did not react with other lipids. Subsequent experiments were therefore conducted only with DGD and NHWM liposomes.

Positive reactions against these were found both in MS patients, normal controls, and patients with amyotrophic lateral sclerosis (ALS), myasthenia gravis (MG) or unspecified neurological disease. However, such reactions were more frequent among MS patients and were quantitatively greater (Table 1). The percentage of lysis in a liposome assay is a quantitative measure analogous to antibody titre⁸, as long as the maximal value obtainable with a specific batch of liposomes (usually about 50%) is not exceeded.

Patients whose sera were reactive did not fall into any specific group with regard to disease classification (progressive, relapsing, or relapsing and progressive), and the degree of lysis obtained was similar when blood was drawn on different dates. Three positive patients were sampled six times and always reacted similarly; negative patients remained so on several subsequent samplings.

The experiments reported above do not support the hypothesis that galactocerebroside or other known galactolipids of human white matter may act as antigens provoking an autoimmune attack on myelin in MS. DGD would appear to be a possible exception. Galactosyl diglycerides, once thought to be exclusively plant lipids, occur in brain, but in no other animal tissues, and are specifically localised in myelin^{13,14}. DGD, in contrast to monogalactosyl diglyceride (MGD), has not been identified with certainty in brain, but it is synthesised by brain microsomes. Both the β -galactose transferase which synthesises MGD and the α -galactose transferase which transforms it into DGD are brain specific, especially abundant in oligodendrocytes, and their activities parallel the process of myelination during development¹⁴.

Table 1 Frequency of reactions against sensitised liposomes

	Digalactosyl diglyceride		Liposomes sensitised with	
	Positives/individuals tested	Mean % lysis* $\pm$ s.d. (positives only)	Positives/individuals tested	Mean % lysis* $\pm$ s.d. (positives only)
MS	60/99	19.6 $\pm$ 19	37/71	8.2 $\pm$ 6
All controls	33/82	10.1 $\pm$ 8†	21/77	5.8 $\pm$ 4†
Normal	18/42		4/33	
Amyotrophic lateral sclerosis	9/26		12/26	
Myasthenia gravis	4/8		3/10	
Unspecified neurological disease	2/6		2/8	

Liposomes were made from distearoyl lecithin, cholesterol and dicetyl phosphate in a molar ratio of 2:1.5:0.2 and contained 10 mM umbelliferyl phosphate in the aqueous phase¹¹. They were sensitised with 150 mg of digalactosyl diglyceride (Applied Science) per μ mol of lecithin, or with a chloroform-methanol (2:1) extract of normal human white matter equivalent to 0.6 mg fresh weight. (In the latter case, the ratio of dicetyl phosphate was increased to 0.4) 1 μ l of the liposome preparation was added to 100 μ l of a complement buffer (20 mM Tris, pH 8.0, with 150 mM NaCl, 0.5 mM MgCl₂ and 0.15 mM CaCl₂) containing 50 μ l per ml guinea pig complement (Grand Island Biological) and 10 μ l per ml alkaline phosphatase. After addition of 5 μ l serum, they were incubated (30 min, 37 °C); 1 ml of the buffer was added before fluorescence measurement.

* %Lysis = $\frac{\text{fluorescence of marker released} \times 100}{\text{fluorescence from total marker entrapped}}$
(Lysis of unsensitised liposomes was subtracted.)

† Significant at $P < 0.005$.

‡ Probably not significant ($P < 0.1$).

Table 2 Absorption of rabbit antisera

Rabbits immunised with:	Normal human white matter	White matter from MS patient % Lysis
Unabsorbed	47.5	48.5
Absorbed with normal white matter	15.5	3.2
MS white matter	14.0	4.2

Liposomes were sensitised with an extract of normal white matter. Rabbits were immunised by injecting 0.5 ml of an emulsion made from 15 mg of lyophilised white matter, 0.25 ml complete Freund's adjuvant and 0.25 ml normal saline (foot-pads and back) and bled 3 weeks later. Sera were absorbed overnight with 30 mg of lyophilised white matter at 4 °C.

If DGD occurs in brain, it is the only known glycolipid which is an α -galactoside¹⁴.

Antibodies of different specificity may be involved in the reaction against DGD and β -galactolipids: (1) rabbit anti-DGD sera do not cross react with cerebroside or gangliosides⁹; (2) many sera which reacted with DGD did not lyse other liposomes; (3) when three reactive sera were absorbed overnight with suspensions of the lipids, only DGD abolished the reaction, while MGD, cerebroside, gangliosides and NHWM were inactive.

Lysis of DGD liposomes may reflect cross reactions with some other glycolipid. An attempt to detect an antigenic difference between the lipids of MS white matter and normal white matter, however, yielded negative results (Table 2).

Circulating antibodies of white matter lipids are likely to be a non-specific phenomenon resulting from the destruction of myelin; they were found in other neurological diseases as well as in normal individuals. Moreover, elevated levels of antibodies to measles and other antigens have been reported in MS (refs 15 and 16). The differences found between MS patients and controls are not sufficient to constitute evidence of a specific antigenic role of DGD in this disease. On the other hand, they would indicate the need to study the occurrence, concentration, localisation and function of this lipid in the nervous system. Our results also illustrate the usefulness of the liposome assay for the detection of antibodies against cell surface glycolipids, which is of interest in cancer immunology.

The cooperation of Drs George W. Ellison and Lawrence M. Myers who provided the human sera is gratefully acknowledged. Our work was supported by grants from the National Institute of Neurological and Communicative Diseases and Stroke and from the National Cancer Institute.

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Effect of botulinum toxin on trophic regulation of acetylcholine receptors

THE distribution of acetylcholine (ACh) receptors in mammalian skeletal muscle is regulated to a large extent by motor nerves¹. In innervated muscles, ACh receptors are localised almost exclusively at neuromuscular junctions, but after denervation there is a great increase of extra-junctional receptor density^{2–5}. The trophic mechanisms by which motor nerves normally control extra-junctional ACh receptor density are not well understood. Although ACh transmission and the muscle usage it produces have been shown to play an important role^{6–11}, it has also been suggested that unrelated factors (for example, substances carried by axonal transport) exert some trophic regulatory influence on extra-junctional ACh receptors^{12–14}. Since more than one factor may participate in this regulation, it is important to evaluate the relative contributions of each in quantitative terms. We have therefore made a quantitative comparison of the effects of botulinum toxin and surgical denervation on ACh receptors. Botulinum toxin blocks quantal release of ACh at nerve terminals¹⁵ in a highly specific manner that is thought to involve the vesicle release mechanism¹⁶. An ¹²⁵I- α -bungarotoxin binding technique was used for quantitative determination of ACh receptor density¹⁷. The results indicate that blockade of ACh transmission by botulinum toxin produces a partial denervation-like increase in extra-junctional ACh receptors, similar to, but somewhat greater than that seen after disuse alone¹⁷.

Injections of botulinum toxin, denervation or control procedures were carried out in 148 female Sprague-Dawley rats (190–215 g) under chloral hydrate anaesthesia (400 mg kg⁻¹). Botulinum toxin (supplied by Dr E. Schantz), freshly diluted in Ringer solution, was injected into the right extensor digitorum longus (EDL) or soleus muscle through a fine No. 30 needle on day 0. Each injection of 30 μ l contained 1.2×10^{-9} g of crystalline type A botulinum toxin¹⁸. For comparison, surgical denervation was produced by avulsing the sciatic nerve approximately 1 cm from the EDL and soleus muscles. Control animals received injections of boiled (inactivated) botulinum toxin. Two further groups of 10 rats were both denervated and injected with botulinum toxin.

At the time of the final experiment (0, 4 and 7 d) the rats were anaesthetised, and neuromuscular transmission was tested in all botulinum-treated muscles. The muscle was removed with the nerve attached, and placed in an oxygenated bath containing Trowell's T8 medium. The nerve was stimulated electrically, using stimulus parameters of 50 V, 100 Hz and 2 ms, and the muscle response was observed under the stereomicroscope. Only muscles that were completely paralysed were used for receptor determinations. Intracellular microelectrode studies were carried out in 17 of these nerve-muscle preparations at various times after botulinum injection. None of the paralysed muscles showed muscle fibre action potentials in response to nerve stimulation, although very small subthreshold endplate potentials (e.p.p.s) (mean amplitude = 0.41 ± 0.07 mV, maximum = 2.5 mV) were recorded intermittently. Neuromuscular blockade was well established within 3–6 h of treatment, and lasted throughout the experimental period.

Miniature endplate potentials (m.e.p.p.s) were recorded

in 26 botulinum-treated muscles. The m.e.p.p. frequency was reduced to 5–10% of normal within 3 h in the soleus and 6 h in the EDL, and rose only slightly by the end of the experimental period (Table 1).

We studied the effects of botulinum treatment on fast axonal transport, since this mechanism has been thought to play a role in neurotrophic regulation^{8,13,19}. Transport of protein and glycoprotein was measured in the sciatic nerves of botulinum-treated rats after injection of ³H-leucine or ³H-fucose into the lumbar ventral horns. In eight rats treated by injection of botulinum toxin 48–144 h previously into all the muscles supplied by the sciatic nerve, the rate of fast transport was normal (383.9 ± 25.4 mm per 24 h)²⁰ and equal to that of the opposite control side. Delivery of ³H-fucose-labelled glycoproteins to the motor nerve endings was also similar in control and botulinum-treated muscles of three rats, as shown by autoradiography of hindfoot lumbrical muscles 24 h after labelling of the ventral horns²¹. Thus the effects of botulinum toxin in these experiments cannot be attributed to interference with either fast axonal transport or delivery of materials to neuromuscular junctions.

Extrajunctional ACh receptor density was measured by a method which utilised saturation binding of ¹²⁵I- α -bungarotoxin^{17,22}. The specific and irreversible binding of this snake venom fraction to ACh receptors allows quantitative measurement of receptor density²³.

The results (Table 2) show that botulinum toxin produced an elevation in extrajunctional ACh receptor density in both the EDL and soleus muscles. The receptor density increased throughout the time studied, being greatest at 7d. This finding is consistent with the earlier observations on extrajunctional sensitivity to iontophoretically applied ACh (ref. 7). The increase in ACh receptors, however, was significantly smaller at the times tested than that produced by denervation (Table 2). Treatment of muscles with denervation plus botulinum toxin resulted in levels of extrajunctional ACh receptors like those of denervation alone. This indicates that the toxin did not inhibit ACh receptors. Furthermore, control injections of inactivated (boiled) toxin did not result in an increase of ACh receptor density, indicating that the experimental procedure itself could not account for the effect of botulinum toxin.

The simplest interpretation of our findings is that the known action of botulinum toxin in blocking neural ACh release is responsible in some way for the observed increase of extrajunctional ACh receptors. Botulinum toxin effectively blocked all suprathreshold ACh release throughout the experimental period, thereby eliminating nerve-induced usage of muscle. In addition, it greatly reduced subthreshold quantal ACh release (m.e.p.p.s and e.p.p.s), as described above. Recent evidence from our laboratory indicates that disuse alone, brought about by local block of nerve conduction, results in a similar but smaller increase of extrajunctional receptors¹⁷. This suggests that the main but

Table 2 Effects of botulinum on extrajunctional ACh receptor density

Treatment		Soleus muscle	
		4 d	7 d
Untreated	22 ± 2 (30)		
Denervation		201 ± 15 (9)*	413 ± 43 (10)*
Botulinum		138 ± 20 (8)†*	278 ± 34 (9)†*
Boiled botulinum		30 ± 10 (3)‡	17 ± 5 (3)‡
Botulinum and denervation			420 ± 35 (5)*§
Extensor digitorum longus			
Untreated	10 ± 1 (36)		
Denervation		219 ± 44 (5)*	420 ± 24 (7)*
Botulinum		42 ± 11 (6)*†	235 ± 48 (6)*†
Boiled botulinum		6 ± 1 (4)†	12 ± 2 (3)†
Botulinum and denervation			435 ± 38 (5)*§

Results are expressed as ACh receptors per μm^2 (mean $\pm$ s.e.m.). Figures in parentheses indicate numbers of muscles.

*Greater than normal $P < 0.001$.

†Less than denervation $P < 0.02$.

‡Less than denervation $P < 0.005$.

§Not different from denervation $P > 0.1$.

not the only denervation-like effect of botulinum toxin on ACh receptors is due to the disuse it produces.

Because the effect of botulinum toxin on extrajunctional ACh receptors was less than that of denervation, some trophic influence must remain unblocked in the botulinum-treated nerves. Many possible factors have been suggested, including substances carried by axonal transport^{8,13,19}, persistent quantal⁷ and non-quantal ACh release, and nerve-muscle membrane interaction^{8,14}. Further studies are needed to clarify the role, if any, of each of these factors.

The effect of cholinergic blockade on ACh receptors does not necessarily parallel its effects on other trophically regulated muscle properties. For example, botulinum treatment completely reproduces the effect of denervation with respect to one such property, isometric contraction of muscle²⁴; but seems to have no effect on another, neuromuscular junction formation in the developing embryo²⁵. Thus, the factors involved in trophic regulation of each muscle property must be evaluated individually.

We conclude that neurotrophic regulation of extrajunctional ACh receptors involves cholinergic transmission, including muscle usage triggered by it. But some additional factor must also play a role.

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Table 1 Effects of botulinum on m.e.p.p. frequency and amplitude

Time	Soleus muscle	
	Frequency*	Amplitude†
0	0.97 ± 0.14	0.38 ± 0.02
3 h	0.16 ± 0.04	0.38 ± 0.05
6–72 h	0.11 ± 0.02	0.28 ± 0.02
7 d	0.16 ± 0.03	0.30 ± 0.04
Time	Extensor digitorum longus	
	Frequency*	Amplitude†
0	2.11 ± 0.37	0.42 ± 0.03
3 h	1.12 ± 0.17	0.33 ± 0.03
6–72 h	0.11 ± 0.02	0.39 ± 0.07
7 d	0.24 ± 0.01	0.43 ± 0.03

*Mean m.e.p.p.s $\pm$ s.e.m. per s.

†Mean m.e.p.p. amplitude $\pm$ s.e.m. corrected to 75 mV RMP.

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Is drug inhibition of dopamine uptake a misinterpretation of *in vitro* experiments?

COYLE and Snyder¹ reported that various antiparkinson drugs inhibit the uptake of labelled dopamine (DA) into striatal synaptosomes. They suggested that these drugs may act in part by a potentiation of dopaminergic neurotransmission caused by inhibition of DA uptake. Measurement of drug effects on the uptake of labelled DA into slices or synaptosomes from the corpus striatum has become a widely used test for the evaluation of drugs affecting central dopaminergic functions. Some observations made in our laboratory, however, suggest that the apparent reduction of ³H-DA uptake caused by a variety of drugs in *in vitro* experiments may, in fact, be the result of a DA-depleting action. Benztropine and nomifensine are said to be potent DA-uptake inhibitors²; however, in our experience, benztropine (30 mg kg⁻¹ subcutaneously) does not inhibit, and nomifensine (100 mg kg⁻¹ orally) only slightly inhibits, the uptake of ³H-DA into striatal synaptosomes prepared from rats pretreated 1 h before killing. In contrast, pretreatment with tricyclic antidepressants does inhibit NA and 5-hydroxytryptamine (5-HT) uptake into midbrain synaptosomes³. Using the method of Farnebo⁴, we studied the effects of benztropine, haloperidol, chlorpromazine, *d*-amphetamine, piroheptine⁵ and cocaine on the release of tritium from field-stimulated, ³H-DA-prelabelled striatal slices. We observed that, at concentrations between 10⁻⁶ M and 10⁻³ M, these drugs increase the spontaneous release of tritium soon after their addition to the superfusion medium.

In the experiments reported here, ³H-DA accumulation *in vitro* was measured in crude synaptosome preparations as described by Coyle and Snyder¹. The corpus striatum of male albino rats of a Sprague-Dawley strain (180–200 g) was homogenised in 0.3 M sucrose solution (1:10 w/v) as described by Whittaker⁶. The homogenate was centrifuged at 1,000 g for 10 min and the supernatant fraction was used. The following components were mixed in this

sequence at 0 °C: 5 ml Krebs-Ringer bicarbonate solution saturated with 95% oxygen, 5% CO₂ (ref. 7), containing per l 100 mg ascorbic acid, 25 mg EDTA and 12.5 mg pargyline; drug to be tested, dissolved in 0.5 ml H₂O; 0.5 ml of the synaptosome suspension; and finally ³H-DA (New England Nuclear, specific activity 3.8 Ci mmol⁻¹), dissolved in 0.5 ml H₂O. The final concentration of ³H-DA was 6 × 10⁻⁹ M and the exogenous amine amounted to ~4% of the total endogenous DA content of the synaptosomes in the incubation. After incubation for 10 min at 37 °C, the synaptosomes were cooled to 0 °C, centrifuged for 15 min at 8,000g, washed with Krebs-Ringer solution, and recentrifuged. The pellet was homogenised in 10% TCA, and the homogenate centrifuged at 12,000g. DA was adsorbed on to alumina at pH 8.6 and eluted with 0.25 N HCl. An aliquot of the extracted catecholamines was counted with 10 ml Insta-Gel, and a second aliquot taken for the automated fluorimetric determination of DA according to the method of Waldmeier *et al.*⁸.

The results of these experiments are summarised in Fig. 1. The following drugs were tested at various concentrations: benztropine, nomifensine, piroheptine, haloperidol, chlorpromazine, diphenylhydramine, clomipramine, cocaine and *d*-amphetamine. In Fig. 1, ³H-DA uptake is plotted against DA content and it can be seen that there was a highly positive correlation between the apparent inhibition of ³H-DA uptake and DA depletion (*r*=0.986).

To see whether the observed phenomenon was restricted to DA uptake into striatal synaptosomes, we compared the effects of several psychotropic drugs on NA content and ³H-NA uptake, using midbrain synaptosomes from rat brain. The method adopted was similar to that used in the experiments with DA and striatal synaptosomes. The added ³H-NA (New England Nuclear, specific activity 4.8 Ci mmol⁻¹, final concentration 6 × 10⁻⁹ M) amounted to about 25% of the total endogenous NA content. The following drugs were tested: desipramine, clomipramine, nomifensine, chlorpromazine, *d*-amphetamine and cocaine and the results are shown in Fig. 2 in the same manner as those of Fig. 1. No depletion of NA was observed with most drugs in concentrations that markedly inhibited NA uptake. Some depletion of NA occurred with very high concentrations (10⁻³ M) of desipramine (28%) and with 10⁻⁶ M *d*-amphetamine (19%). The depleting action of *d*-amphetamine can be explained by the well known NA-releasing effect of this drug, observed *in vivo* in experiments performed with the push-pull cannula technique^{9,10}.

Fig. 1 Comparable effects of psychotropic drugs on ³H-DA uptake and endogenous DA content in rat striatal synaptosomes after 10 min incubation at 37 °C. Estimations of uptake and content were carried out in each case in the same synaptosome suspension. Each point represents the mean of 2–4 estimations. Ordinate: ³H-DA uptake as a percentage of the corresponding control values (80,000–100,000 d.p.m. per sample, tissue: medium ratio for control ³H-DA uptake, 80–100:1). The value obtained in the presence of 10⁻³ M benztropine (37 °C) was taken as nonspecific background uptake (about 6% of the uptake of the control). Abscissa: Endogenous content of DA as a percentage of the corresponding control values. The absolute values of the same controls as used for determination of ³H-DA uptake ranged between 130 and 170 ng DA per sample.

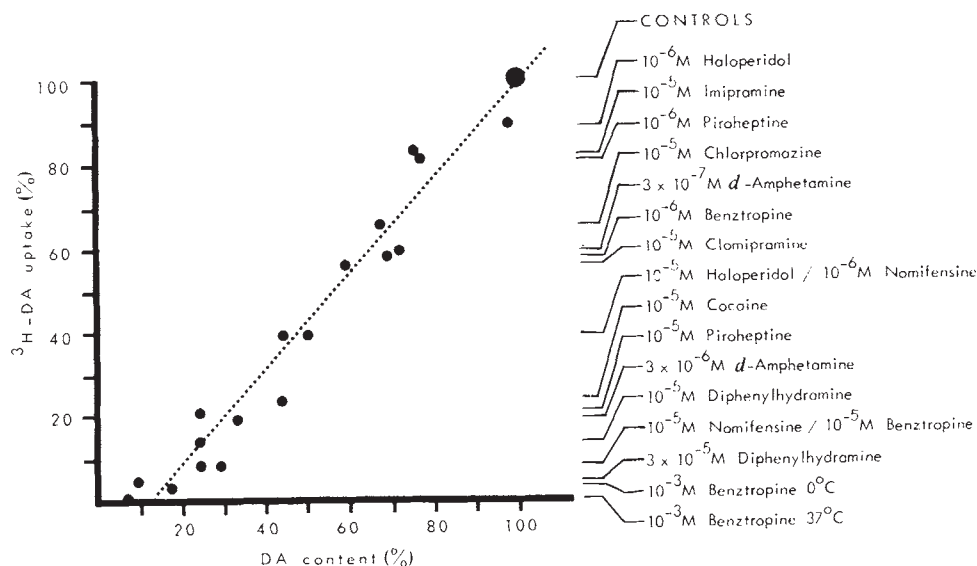
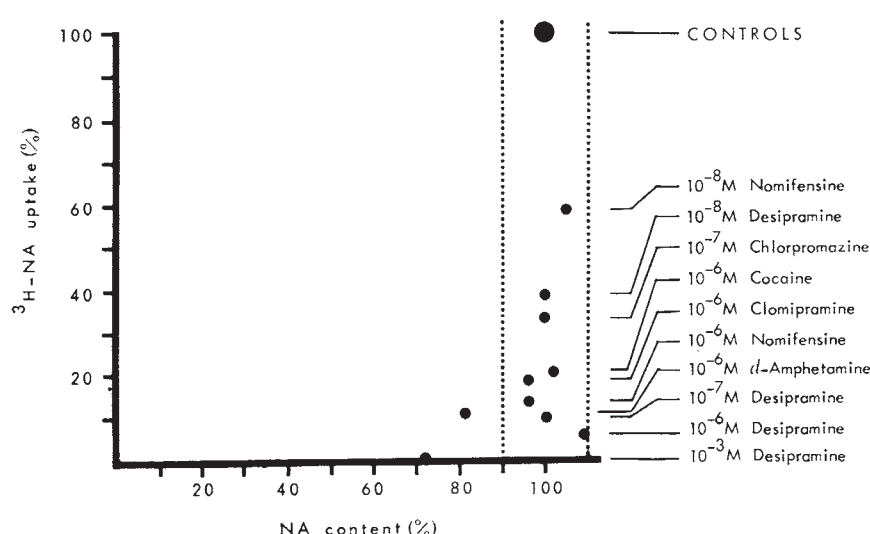


Fig. 2 Comparable effects of psychotropic drugs on ^3H -NA uptake and endogenous NA content in synaptosomes from rat mid-brain (including diencephalon) after 30 min incubation at 37 °C. Estimations of uptake and content were carried out in the same synaptosome suspension. Each point represents the mean of two estimations. Ordinate: ^3H -NA uptake as a percentage of the corresponding control values (21,000 d.p.m. per sample, tissue: medium ratio for control ^3H -NA uptake, 17:1). The values obtained in the presence of 10^{-3} M desipramine was taken as unspecific background uptake (about 15% of the uptake of the control). Abscissa: Endogenous content of NA as a percentage of the corresponding control values. The absolute value of the same controls as used for determination of ^3H -NA uptake was 22.5 ng NA per sample.



This effect was also observed *in vitro* in our laboratory with 10^{-6} M *d*-amphetamine in experiments in which superfluous ^3H -NA-prelabelled cortical slices were used.

All the drugs commonly assumed to be DA-uptake inhibitors that we have tested so far have proved to be effective in releasing endogenous DA from striatal synaptosomes. Our data support the suggestion of Orlansky *et al.*¹¹ and Heikkilä *et al.*¹² that the so-called DA-uptake inhibitors are in fact DA-releasing agents, and that the apparent inhibition of DA uptake can be explained by drug-induced depletion of DA stores *in vitro*. They also bear out these earlier conclusions that a drug can only be designated as a DA-uptake inhibitor if a depleting effect has been excluded. On the other hand, it seems that the uptake of ^3H -NA into synaptosomes or slices from parts of the brain other than the striatum is a valid model for measuring NA-uptake inhibition.

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Release of substance P from isolated nerve endings

DISCOVERED in 1931 by von Euler and Gaddum¹ and purified to homogeneity in 1970 by Chang and Leeman², substance P is an undecapeptide with the sequence H-Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (ref. 3). Its function is still unknown but the selective distribution of substance P in the nervous system^{4–6}, its localisation in

nerve endings (refs 7–9 and our own unpublished results) and its neuropharmacological actions^{10–14} strongly suggest that substance P may participate, directly or indirectly, in the transmission process of certain neurones. However, if substance P is a neurotransmitter, it must also be released from nerve terminals upon the appropriate stimulation. In this report we present evidence showing that substance P is released from isolated nerve endings by depolarisation with high K⁺ and that this release is calcium dependent.

These experiments were performed on synaptosomes, a preparation which has been useful in the study of the release of other putative neurotransmitters in the central nervous system¹⁵. Synaptosomes or crude synaptosomal (P₂) pellets were prepared according to Scheme II of Cotman¹⁶, starting with hypothalami and ventral mesencephalons of adult male rats (Charles River Breeding Co., Wilmington, Massachusetts). The tissue was placed on a filter and superfused with a Ca²⁺-free physiological salt solution. After the initial washout, the synaptosomal tissue was periodically exposed to brief pulses of test solutions. The superfusates were collected and the substance P content determined by radioimmunoassay¹⁷. In our initial studies we found no difference between synaptosomal fractions and crude synaptosomal (P₂) pellets with regard to their pattern of substance P release. Furthermore, none of the remaining fractions of the sucrose-Ficoll gradient used to prepare synaptosomes contained appreciable amounts of substance P. All data presented here were obtained with crude synaptosomal (P₂) pellets.

Since synaptosomes have a K⁺ diffusion potential, depolarisation can be achieved by superfusing them with a high K⁺ medium¹⁸. Only in the presence of Ca²⁺ does exposure to 60 mM K⁺ induce a significant release of substance P (Fig. 1). This is analogous to the conditions that have been described for the release of established and putative transmitters as well as for several hormones¹⁹. Within our time resolution the evoked release of substance P occurs immediately and subsides after the removal of the stimulus (Fig. 2). It is interesting to note that a second stimulating pulse, administered after a brief interval, invariably released less substance P than the first (Fig. 2) and a third less than the second (not shown). This reduction in release is not due to a K⁺- or time-dependent deterioration of the tissue since prior exposure to high K⁺ in the absence of Ca²⁺ had no effect on the evoked release and since a stimulating pulse was as effective at the end of an experimental run as at the beginning (see legend to Fig. 1). The decreased release may indicate a limited size of the substance P pool available for release or the gradual

inactivation of the Ca^{2+} dependent depolarisation-secretion coupling process giving access to that pool.

Microsomes, normally remaining in the supernatant when a crude synaptosomal pellet is obtained²⁰ sometimes contaminate the P_2 pellets. Because an appreciable amount of substance P is associated with this fraction (ref. 9 and our own unpublished results), we tested it for release of substance P. The small amount retained on the filters could not be released by high K^+ in the absence of Ca^{2+} . We

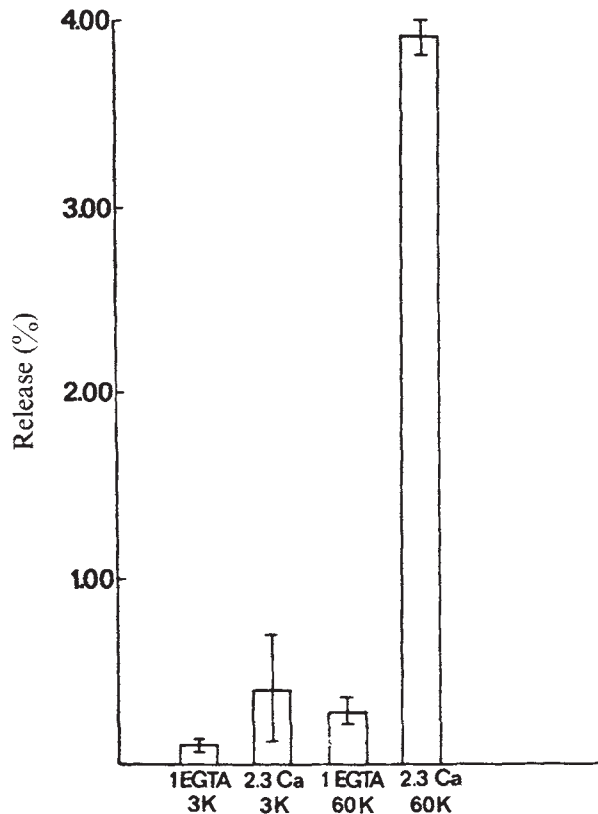


Fig. 1 Release of substance P from superfused synaptosomes. Synaptosomes were placed on filters (Nucleopore N80 CPR, Millipore AP20 prefilter) and superfused for 5 min at 5 ml min^{-1} , thereafter at 10 ml min^{-1} with EGTA+3K (for ionic composition see below). After the baseline efflux had stabilised, pulses of test solutions (see below) were administered for 36 s at 2.4-min intervals in varying sequence. The 2.3 Ca+60K pulse was usually given at the end of a run since it released a considerable portion of the substance P available for release (see Fig. 2). Fractions of the superfusates were collected every 18 s into acetic acid. The release studies were carried out at room temperature ($20\text{--}23^\circ\text{C}$). At the end of an experimental run the filters retaining the tissue were extracted in 2N acetic acid. Superfusates and filter extracts were lyophilised and the substance P content determined by radioimmunoassay. Each bar in the histogram represents the amount of substance P released during 36 s in response to the respective pulse. The base-line efflux (EGTA+3K) was obtained from the means of the 3 fractions preceding a given pulse. The amount of substance P on filters at time of collection of a given sample was estimated from the amount of substance P on the filter at the end of an experimental run plus the cumulative amount of substance P collected in the effluent. Values are expressed as: amount of substance P collected/estimated amount of substance P on filter at time of collection $\times 100$. Mean $\pm$ s.e.m. ($n = 4$; for EGTA+3K, $n = 20$; for 2.3 Ca+60K, $n = 6$). Physiological solutions: An artificial cerebrospinal fluid, buffered with HEPES at pH 7.3 and oxygenated with O_2 was used. When necessary, Na^+ was replaced isotonicly by K^+ , and/or Mg^{2+} was replaced by Ca^{2+} . The basic composition ("2.3 Ca+3K") was as follows: 135 mM NaCl, 3 mM KCl, 2.3 mM CaCl_2 , 2.3 mM MgCl_2 , 0.5 mM Na_2HPO_4 , 10 mM glucose, 20 mM HEPES and 0.2% bovine serum albumin. "2.3 Ca+60K" contained 60 mM KCl, "EGTA+3K" contained 4.6 mM MgCl_2 and 1 mM EGTA. "EGTA+60K" contained 4.6 mM MgCl_2 , 1 mM EGTA and 60 mM K^+ .

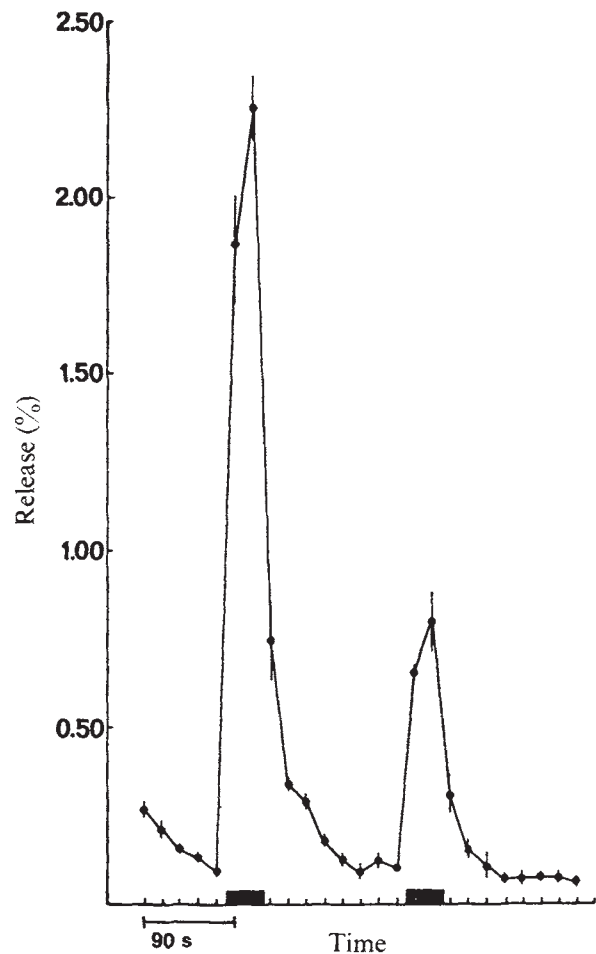


Fig. 2 Time course of release of substance P. Experiments were carried out as described in Fig. 1. Synaptosomes were superfused with EGTA+3K except at bar, where they were exposed to 2.3 Ca+60K for 36 s. Each time division is 18 s. Values expressed as: amount of substance P released/amount of substance P on filter at time of collection $\times 100$. Mean $\pm$ s.e.m. ($n = 3$).

are therefore confident that the substance P in crude synaptosomal pellets was released from nerve endings and not from any contaminants.

Recently, Otsuka has also reported that the substance-P like immunoreactivity in the perfusate of the isolated spinal cord of new-born rats is increased by soaking the preparation in a solution containing 55 mM K^+ (ref. 11).

These results show qualitatively that substance P can be released in a manner similar to that of other suspected neurotransmitters. Although this finding can be taken to support a transmitter role for substance P, some important quantitative questions remain to be answered. First, the precise amount of substance P needed to mimic the physiological activity of a given synapse, including the time course of that activity, must be determined. Second, the timing and amount of substance P released at that synapse must be determined and shown to be adequate to account for the normal activity of that synapse. Until such quantitative information is obtained the question of whether substance P is a transmitter in the nervous system remains open. In any case, the possibility that substance P may have other functions, such as the modulation of synaptic activity at a pre- or postsynaptic site, should not be neglected.

Since submission of this manuscript, the release of

immunoreactive substance P from an isolated spinal cord preparation of new-born rats²¹ and rat hypothalamic slices²² has been reported.

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Induction of tolerance to the analgesic action of lipotropin C-Fragment

THE C-Fragment of β -lipotropin (lipotropin 61-91), was first discovered as an intact peptide in the pituitary gland^{1,2} and it has recently been shown to be present in brain³. The peptide binds strongly to brain opiate receptors *in vitro*^{4,5} and has potent opiate-like actions on the guinea pig ileum and mouse *vs* deferens¹⁵. Reports from our laboratory described the potent analgesic properties of C-Fragment when injected into the cerebral ventricles of the cat⁴⁻⁶, and in subsequent studies its analgesic effects have been demonstrated in rodents⁷⁻⁹. The results reported here confirm the analgesic potency of C-Fragment administered intracerebrally in the rat, and show that chronic administration of

the peptide leads to the development of tolerance to its analgesic actions.

C-Fragment was isolated from pig pituitary glands as described previously². Cannulae were implanted in the lateral ventricles of male Wistar rats weighing 150-170 g¹⁰. After a recovery period of at least 1 week, response latencies of the animals were measured on a hot plate (54.2 $\pm$ 0.2 °C) according to Eddy and Leimbach¹¹. The criterion of reaction of the rat was licking of one paw, or intensive jerking with lifting off or jumping on the hind legs. The trial was terminated if response latency exceeded 60 s.

Two experiments were carried out, using the same schedule of chronic peptide administration: groups of 6 rats received intracerebral injections of 4.5 μ g C-Fragment (1.5 μ g μ l⁻¹, dissolved in 1 M NaCl) or of placebo (3 μ l of 1 M NaCl) twice daily (at 9.30 a.m. and 5.30 p.m.) for 2 d.

Experiment 1 was designed to detect first the onset of tolerance to the analgesic action of C-Fragment, and second the possible development of cross-tolerance to the action of peripherally-administered morphine. A group of 6 rats was treated with C-Fragment according to the schedule described above. Results from 2 animals were later discarded, owing to improper location of the cannulae. A second group of 6 animals was treated with placebo. The acute analgesic action of the peptide was established by measurement of hot plate response latency before and after the first injection of peptide and placebo.

Response latency underwent a large increase in the C-Fragment treated rats (Fig. 1a). The time course of action was fairly rapid; analgesic activity was maximal 20 min after injection, and faded rapidly thereafter. Acute injection of lower doses of the peptide (0.3 and 1.1 μ g) did not significantly increase response latency, indicating that the dose-response curve is rather steep.

At 9.30 a.m. on d 3 of the experiment, both groups of animals received an injection of 4.5 μ g C-Fragment. Hot plate response latencies were monitored both before and after injection, and were compared with corresponding latencies measured for a group of 6 control animals which were given a single acute intracerebral injection of placebo. Forty minutes after the intracerebral injection, the three groups of animals were given a single i.p. injection of 10 mg kg⁻¹ morphine-HCl, and hot plate response latencies were measured 30 min later. The results of these measurements are summarised in Fig. 1(b).

Chronic pretreatment with placebo had no effect on the analgesic response to C-Fragment. The response latencies of animals chronically pretreated with the peptide, however, underwent only a slight increase. This increase was significantly less than that obtained with the placebo-pretreated group ($P < 0.05$, Wilcoxon test). In every case the increases in response latencies of individual animals which had been chronically pretreated with C-Fragment were lower than the increases measured for the same animals after the first injection of the peptide. This indicated that tolerance had developed to the analgesic action of the peptide.

Systemic administration of morphine evoked similar increases in response latency in all three groups of animals (Fig. 1b). There

Fig. 1 Mean response latency as assessed on the hot plate of animals treated intracerebrally with 4.5 μ g C-Fragment twice daily (4 animals, ●) or with placebo twice daily and on d 3 with 4.5 μ g C-Fragment (6 animals, ■). Morphine-HCl (10 mg kg⁻¹ was administered intraperitoneally (m.) 40 min after the last injection with C-Fragment. One group of 6 animals (▲) received an intracranial placebo injection on d 3. Results are given as mean $\pm$ s.e.m. Unless otherwise indicated, standard errors are within the size of the points.

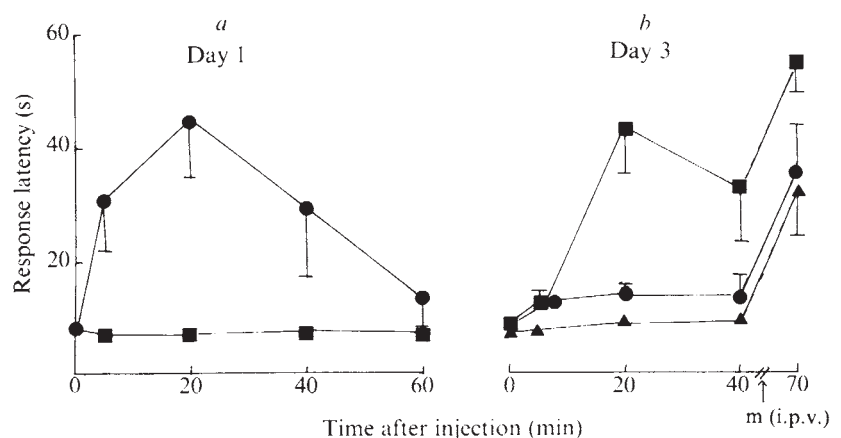
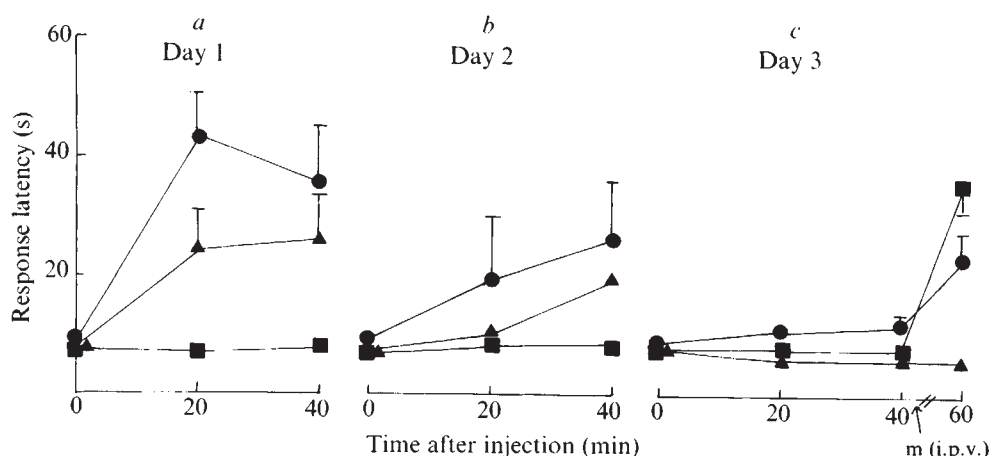


Fig. 2 Mean response latency as assessed on the hot plate of animals treated intraventricularly with 4.5 μ g C-Fragment (5 animals, $\bullet$) or with placebo (6 animals, $\blacksquare$) twice daily. One group of 6 animals treated with C-Fragment received Pro-Leu-Gly-NH₂ (1 μ g per animal, subcutaneously) 1 h before C-Fragment on the d 1 only ($\blacktriangle$). On d 3 morphine-HCl (15 μ g per animal) was administered intraventricularly (i.v.) 40 min after the injection with C-Fragment. Results are given as mean $\pm$ s.e.m. Standard errors are within the size of the points, unless otherwise indicated.



was therefore no sign of cross-tolerance to the narcotic after peripheral administration. However, it is notable that the level of analgesia attained after morphine injection in animals which had been chronically pretreated with placebo followed by a simple injection of C-Fragment was substantially greater than that found in the C-Fragment-pretreated or control animals, indicating a probable potentiation of the analgesic effects of morphine by a single dose of the peptide, an effect which seems to disappear after chronic treatment.

Experiment 2 was designed first to examine the possible development of cross-tolerance between the analgesic effects of C-Fragment and intracerebrally administered morphine, and second, to examine the effects of pretreatment with the C-terminal tripeptide of oxytocin, pro-leu-gly-NH₂ (PLG) on the time-course of tolerance development, and on the degree of cross-tolerance developed to morphine. It has been shown previously that pretreatment with oxytocin, or PLG, markedly facilitates the development of morphine tolerance and physical dependence in the rat¹².

Three groups of animals were used in this experiment. Group 1 (5 animals) received a single subcutaneous injection of 1 μ g of PLG dissolved in 0.2 ml saline, and groups 2 and 3 (6 animals) control injections of 0.2 ml saline 1 h before the first injection of C-Fragment. Groups 1 and 2 were given intracerebral injections of 4.5 μ g C-Fragment, and group 3 injections of 1 M NaCl according to the schedule previously described. Hot plate latencies were tested before and after the first and third injections, on d 1 and 2 respectively. On d 3, groups 1 and 2 were again injected with C-Fragment, and group 3 with placebo, and hot plate latencies assessed. 40 min after the final injections of C-Fragment and placebo, all 3 groups of animals were given an intracerebral injection of 15 μ g morphine-HCl. Hot plate latencies were monitored 20 min later. The results of this experiment are summarised in Fig. 2.

Animals pretreated with C-Fragment alone again developed tolerance to the analgesic action of the peptide. This was clearly visible on d 2 (Fig. 2b), and virtually complete on d 3 (Fig. 2c). Pooling the results from experiments 1 and 2, increases in response latencies were significantly smaller on day 3 compared with day 1 both 20 and 40 min after C-Fragment injection ($P < 0.01$, $P < 0.05$ respectively, Wilcoxon signed rank test, 9 animals).

Pretreatment with PLG appeared to exert some depressant effect on the acute antinociceptive action of C-Fragment (Fig. 2a). However, this effect was not statistically significant ($P > 0.1$, Wilcoxon test). Pretreatment with the tripeptide tended to accelerate the development of tolerance to C-Fragment (Fig. 2b and c), but a larger number of animals will be needed to establish this effect with more certainty. However, the PLG-treated animals, which had been chronically injected with C-Fragment showed complete cross-tolerance to the analgesic effect of an intraventricular injection of 15 μ g of morphine-HCl (Fig. 2c). Morphine injection increased mean response latency by 30 s in the placebo-pretreated group, and by 11 s in the group pretreated with C-Fragment alone.

The difference between these values is marginally significant ($P < 0.1$, Wilcoxon test). However, the response latency of the PLG-treated group failed to increase after morphine injection, in any of the animals tested ($P < 0.005$ with respect to both of the other groups). These findings suggest that chronic pretreatment with C-Fragment induces cross-tolerance to morphine. PLG accelerates the development of cross-tolerance and therefore presumably enhances the development of tolerance to the analgesic action of peptide itself.

The results of these studies confirm the high analgesic potency of C-Fragment in the rat. On a molar basis, the analgesic potency of the peptide injected into the lateral ventricle is 30–40 times that of morphine, in good agreement with the findings of Loh *et al.*⁷, and Graf *et al.*⁸. A comparable potency ratio has been reported for opiate-like effects of the peptide after injection into the periaqueductal grey matter¹³.

Chronic administration of C-Fragment leads to the development of tolerance to its analgesic effects, and there are strong indications of the development of some cross-tolerance to intracerebral morphine. The absence of cross-tolerance to peripherally administered morphine must be interpreted with caution, firstly because in animals pretreated with C-Fragment alone, cross-tolerance to intracerebral morphine was not complete, and secondly because the population of receptors involved in mediating the antinociceptive effects of relatively low peripheral doses of morphine may not be identical to that occupied after intracerebral injection¹⁴.

The findings reported here accord with the ability of C-Fragment, and other opiate peptides to induce morphine-like physical dependence¹³, and suggest two intriguing possibilities: that mechanisms involved in the production of tolerance may be important in regulating the actions of endogenous opiate peptides *in vivo*, and that other neuropeptides may play a part in modulating these processes.

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Induction of excessive grooming in the rat by fragments of lipotropin

CURRENT research reveals the existence of endogenous peptides in brain^{1,2} and pituitary tissue^{3,4}, which are presumably derived from lipotropin (LPH) and which have opiate like effects and affinity for opiate receptors (enkephalin¹, endorphins⁵, C-Fragment⁶). N-terminal peptides of ACTH also have measurable, although much lower, affinity for rat brain receptors *in vitro*^{7,8}. ACTH and congeners are known to play a crucial role in the acquisition and maintenance of a variety of behaviours in animals and man^{9,10}. Intraventricular but not systematic administration of these peptides elicits a stretching and yawning syndrome^{11,12}. In rats this syndrome is preceded by a display of excessive grooming^{13,14} and this grooming response can be suppressed by peripheral administration of specific opiate antagonists (naloxone, naltrexone)¹⁵. Morphine also induces grooming¹⁵ and this observation prompted us to study the effect of LPH fragments on excessive grooming in the rat in the absence and presence of an opiate antagonist.

The methodology has been described in detail elsewhere^{14,15}. Briefly, female rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) had plastic canulae implanted in the 3rd cerebral ventricle, 1 week before the observation session. The behavioural procedure consisted of a 15th-s sampling technique, the validity of which has already been established¹⁴. During a 50-min period the observer determined every 15th s whether the rat displayed an element of the maintenance repertoire (that is vibrating, washing, grooming, scratching, licking paw, licking tail)¹⁶. Since the predominant element recorded seemed to be grooming, we prefer to refer to grooming in keeping with previous reports^{11,13,14}. Immediately after intraventricular injections into conscious rats (1 μ l), the rats were placed individually into glass boxes (24 $\times$ 12.5 $\times$ 14 cm) in a low noise room and 15 min later the behavioural analysis began. The effect of naloxone (Endo, Ny, 0.5 ml 100 g⁻¹ subcutaneous 1 mg kg⁻¹) on peptide-induced excessive grooming was studied 25 min after the intraventricular injection of the peptide. Therefore the scoring was interrupted for 5 min to allow for administration of the opiate antagonist; subsequently the behavioural analysis was continued for another 40 min. The following synthetic peptides were tested: ACTH₁₋₂₄, β -MSH (LPH₄₁₋₅₈), ACTH₄₋₁₀ (LPH₄₇₋₅₃), Met-enkephalin (LPH₆₁₋₆₅), Leu-enkephalin ([Leu⁶⁵]LPH₆₁₋₆₅), LPH₆₁₋₆₉, LPH₆₅₋₆₉, α -endorphin (LPH₆₁₋₇₆), C-Fragment (LPH₆₁₋₉₁) was isolated from porcine pituitary glands³.

Saline treated rats, placed in a novel glass box usually display exploratory and grooming behaviour which may last for some 15–20 min after intraventricular injection but then invariably fall asleep and thus show little grooming activity during the observation period, which starts 15 min after the injection. In contrast, the intraventricular injection of 0.3 μ g of LPH₆₁₋₉₁ elicited nearly maximal grooming activity (165 out of 200 possible positive scores). The effect appeared to be dose-dependent. A dose of as little as 10 ng significantly induced grooming activity (Table 1). LPH₆₁₋₉₁ therefore has a higher potency than ACTH₁₋₂₄¹⁴. It should be noted, however, that in contrast to ACTH-induced grooming, administration of LPH₆₁₋₉₁ in low doses such as those used here, not only elicited grooming but also excitation in some

rats typified by quick movements of body and head, jumping, gnawing and body shakes. In a subsequent experiment the effect of subcutaneously administered naloxone on LPH₆₁₋₉₁-induced excessive grooming was studied. Rats treated with 0.1 μ g LPH₆₁₋₉₁ displayed excessive grooming before naloxone (Table 2). However, after subcutaneous administration of 1 mg kg⁻¹ naloxone, excessive grooming was markedly suppressed. Interestingly, no overall behavioural differences were noted between saline/saline and saline/naloxone treated rats, suggesting that naloxone itself did not affect ongoing behaviour. Similar results were obtained using 0.1 mg naloxone and 0.1 μ g LPH₆₁₋₉₁. Structure activity studies were carried out to identify the active amino acid sequence responsible for excessive grooming. It has been found previously that intraventricular injection of sheep β -LPH does not elicit grooming in the rat unless an extreme dose (2.5 mg) is used¹³. Apparently β -LPH itself is practically devoid of activity in this respect. LPH₆₁₋₇₆ (α -endorphin) was much less active than LPH₆₁₋₉₁. The sequence LPH₆₁₋₆₉ seemed slightly less active than LPH₆₁₋₇₆ (Table 1). LPH₆₁₋₆₅ and [Leu⁶⁵]LPH₆₁₋₆₅ induced hardly any grooming activity over the wide dose range tested (0.1–29 μ g). Similarly, LPH₆₅₋₆₉ was found to be inactive (Table 1). LPH₄₇₋₅₃ which is common to LPH, MSH and ACTH has latent grooming activity¹⁷. Administration of LPH₄₇₋₅₃ in doses up to 40 μ g does not induce excessive grooming (Table 1) whereas administration of LPH₄₇₋₅₀ or [D-Phe⁵⁰]LPH₄₇₋₅₃ (= [D-Phe⁷]ACTH₄₋₁₀) elicits significant grooming at a dose of 1 μ g^{14,15}. As reported previously the sequence LPH₄₁₋₅₈ (= β -MSH) is equipotent to ACTH₁₋₂₄ in inducing the grooming response (Table 1)¹⁴. Excessive grooming was interrupted by short episodes of stretching and yawning only in the case of ACTH₁₋₂₄, LPH₄₁₋₅₈ and LPH₆₁₋₇₆, again suggesting that excessive grooming behaviour and stretching/yawning may result from different mechanisms¹⁴. The results indicate that within the C-fragment LPH₆₁₋₆₉ contains the essential sequence for excessive grooming. Elongation of this sequence, however, markedly enhances the response.

The available data on the affinity of LPH fragments for opiate receptors in rat brain membrane fractions indicate that LPH₆₁₋₉₁ has the highest affinity (IC₅₀ for DHM 2.2×10^{-9} M, for etorphin 3×10^{-7} M^{5,6}) followed by (61–65), (61–69) and (61–76) in that order. The affinity of fragments of ACTH and LPH₄₇₋₅₃ (ACTH₄₋₁₀) studied with labelled DHM is several orders of magnitude lower than those peptides mentioned above and the sequence α -MSH [Ac-Ser³]ACTH₁₋₁₃-NH₂—which is as active as ACTH₁₋₂₄ in inducing excessive grooming¹⁴—is virtually inactive^{7,8}.

Recently, we have reported that subcutaneously administered fragments of ACTH which have some affinity for the brain opiate receptors and which are devoid of *in vivo* corticotrophic activity counteract morphine-induced analgesia in the rat as tested on the hot plate^{18,19}.

The biological significance of induction of excessive grooming is unclear. Some authors have interpreted the behavioural signs

Table 1 LPH fragments and excessive grooming

Peptide sequence	Lowest dose at which excessive grooming was observed* (μ g)
LPH ₆₁₋₉₁	0.01
LPH ₆₁₋₇₆	1.7
LPH ₆₁₋₆₉	5.5
LPH ₆₁₋₆₅	>29†
[Leu ⁶⁵]LPH ₆₁₋₆₅	>29†
LPH ₆₅₋₆₉	>20†
LPH ₄₁₋₅₈ (= β -MSH)	0.1
LPH ₄₇₋₅₃ (= ACTH ₄₋₁₀)	>40†
ACTH ₁₋₂₄	0.1

*Grooming was scored using a 15th-s sampling technique (observation session of 50 min, beginning 15 min after intraventricular injection). For all peptides at least 5 dosages were tested with at least 4 rats per group. Saline-treated rats as average reach a score of 20–30 (out of maximum of 200). Significant excessive grooming is observed when scores are above 55.

†No activity observed over a dose range of 0.1 μ g to indicated dose.

Table 2 Effect of naloxone on LPH₆₁₋₉₁ induced excessive grooming in the rat

		Treatment		Number of rats	Amount of grooming	
<i>t</i> = 0 min IIIrd ventricle		<i>t</i> = 25 min subcutaneous			15–25 min	30–70 min
LPH _{61–91}	0.1 µg	saline	0.5 ml	6	32 ± 1*	87 ± 12
LPH _{61–91}	0.1 µg	naloxone	1 mg/kg	6	29 ± 2	6 ± 3
saline	1 µl	naloxone	1 mg/kg	3	14 ± 8	12 ± 7
saline	1 µl	saline	0.5 ml	3	16 ± 6	9 ± 5

*Mean ± s.e.m.

induced by intracranial administration of ACTH-like peptides in terms of sexual excitement^{20,21}. The behavioural response in pigeons has been likened to displacement behaviour²². Both electrical stimulation of limbic structures^{23,24} and central application of Zn²⁺ (ref. 12) may induce grooming activity. The latter observation is of interest in view of the known importance of bivalent cations to the mechanism of action of ACTH^{25,26}.

The present study was aimed at investigating the nature of the peptide-CNS interaction underlying behavioural effects. Since opiate antagonists suppress ACTH and LPH-induced behaviour, one is tempted to conclude that the neural substrate for this behaviour is sensitive to ACTH fragments, LPH fragments and opiates. Interestingly, intraventricular administration of low doses of morphine also induces excessive grooming to the same extent as LPH₆₁₋₇₆¹⁵.

In view of the behavioural effects produced by intracerebral injection of low doses of LPH fragments, the question whether the "opiate-like" activity of these neuropeptides is their most important physiological effect remains to be elucidated.

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Neonatal progesterone and feminine sexual development

It has been generally concluded that the inherent programme of sexual differentiation in both sexes of mammals is female. If androgens are present during the critical periods of sexual differentiation, then both genetic males and females will be organised for masculine reproductive organs¹, hepatic steroidogenic enzymes², hypothalamic control of gonadotropin secretion (tonic)³ and sexual behaviour⁴; whereas an absence of either gonad during the critical developmental periods allows for the expression of the inborn female programme^{1-3,5}. These results have led to the generally held concept that feminine differentiation requires no hormonal imprinting and will occur normally as long as androgens are not present during the critical periods of sexual embryogenesis. We have reported, however, that interference with perinatal pituitary or adrenal function in female rats causes defects in normal pubertal feminine development which suggests that endogenous hormones may be essential for feminine organisation⁶. Unlike oestradiol and testosterone which both masculinise the female rat⁷, progesterone treatment antagonises these effects and protects the developing female from exogenous oestrogens and androgens⁸. In fact, serum progesterone levels in the foetal monkey have been shown to be significantly higher in the female than in the male⁹ and we have recently postulated that perinatal progesterone may be required for feminine neural differentiation¹⁰. We present here evidence demonstrating that neonatal female rats have a markedly higher level of serum and adrenal progesterone than do neonatal males, and since serum progesterone levels can be further increased by exogenous gonadotropins-adrenal (progesterone) axis may be required for normal feminine sexual differentiation.

At 3 d of age the serum progesterone concentration was ten times greater in female pups than in males (Table 1) and the adrenal progesterone concentration was also significantly greater in the neonatal females. Administration of pregnant mare serum gonadotropin (PMSG, which has both follicle stimulating hormone (FSH) and luteinising hormone activities, with FSH predominant¹¹) elevated serum progesterone levels on both 3-d-old male and female rats.

Our progesterone results in neonatal rats are in agreement with findings in adult animals, in that rat adrenals

have been shown to secrete progesterone¹² and this secretion is selectively enhanced by gonadotropins¹³. Furthermore because the ovary of the rat is steroidogenically inactive^{14,15} and unresponsive to gonadotropins^{16,17} until the second week of life, it seems certain that the neonatal adrenal was the source of serum progesterone, at least in the female. Although the neonatal testis is steroidogenically competent¹⁴, and secretes androgens²⁻⁵, the presence of significant levels of progesterone in the male adrenals suggests that they may also be the source of serum progesterone in the neonatal male.

It now seems possible to describe a functioning sexual neuroendocrine axis in the neonatal female rat. The hypothalamus of the newborn female rat contains increasing levels of gonadotropin releasing hormone (GnRH)¹⁸, and the pituitary of the newborn female rat is more sensitive than that of the male to GnRH, and thus secretes greater quantities of LH in response to GnRH treatment¹⁹. This enhanced sensitivity to GnRH by the female pituitary may be the cause of the much higher levels of serum LH and FSH found in newborn females as compared with males¹⁸⁻²¹. These elevated gonadotropin levels in newborn females may, in turn, stimulate adrenal hormone production resulting in higher serum progesterone levels in the female. There is thus a distinctive sexually dimorphic character in the functioning of the hypothalamic-pituitary-adrenal axis of the neonatal female rat which clearly suggests a role for perinatal hormones in feminine sexual development.

The elevated progesterone levels in the newborn female are of particular importance. During the first few days of life both male and female rats have very high levels of serum oestrogens^{15,21} and androgens²¹, and it is during this period that the brain is most sensitive to the masculinising effects of testosterone and oestradiol⁷. The raised serum progesterone in the newborn female may function as a hormone antagonist and protect the developing female brain from the masculinising actions of the endogenous

oestrogens and androgens and thus, allow for differentiation of the inborn female development pattern. It is also possible, however, that progesterone has a direct effect on female organisation and that feminine differentiation is not a result of an inherent developmental programme, but requires active imprinting by perinatal hormones, such as adrenal progesterone¹⁰.

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Table 1 Progesterone levels in 3-d-old rat pups treated with PMSG

		Serum progesterone (pg ml ⁻¹)	
	No. of pups	Control	PMSG
Male	23	49.9 ± 7.0*	467.9 ± 59.3§
Female	16	444.6 ± 58.6§	604.9 ± 43.1†

		Adrenal progesterone (pg mg ⁻¹)	
		Control	PMSG
Male	21	465.1 ± 48.1	448.6 ± 75.3
Female	12	762.4 ± 107.7‡	603.9 ± 58.4

Within 6 h of parturition half the male and half the female pups in each litter were injected with 16 IU PMSG (NIAMD) and the other half received the diluent, 4 µl of 0.9% NaCl. The same treatment was repeated 24 and 48 h after the first injections. The pups were decapitated on the third day of life, 6-8 h after the third injection. Blood was collected in microcentrifuge tubes and adrenal glands were quickly removed, cleaned, weighed and frozen. None of the serum or adrenals was pooled for the assay, and although adrenal progesterone was determined in duplicate analyses, the small quantities of serum allowed for only single determinations. Progesterone was measured by radioimmunoassay using purchased antibodies (Miles-Yeda) produced by immunising rabbits with progesterone-11α-hemisuccinyl-bovine serum albumin. The progesterone assay was similar to reported procedures²² with the exception that serum and adrenal homogenates were extracted with petroleum ether and the chromatographic step was omitted because the only significant cross reactivity was with 11β-hydroxyprogesterone at only 8%. Recovery from extracted serum using ³H-progesterone ranged from 68 to 73%. The sensitivity of the assay allowed for the detection of 5 pg of progesterone per tube. In ten duplicate determinations the coefficient of variation was 6.3%.

* Mean ± s.e.

Statistical analysis by Student's *t* test comparing control males with control females and controls with PMSG of the same sex.

† *P* < 0.03; ‡ *P* < 0.01; § *P* < 0.001.

Spermatogenesis and 3β-HSDH activity in the testis of the axolotl

THERE has long been considerable debate over the functions of various hormones in promoting or maintaining spermatogenesis. Much of the work in this field has been carried out on mammals, where interpretation is especially difficult because of the heterogeneous content of the seminiferous tubules¹. Although a more favourable situation prevails in some sub-mammalia as lower amphibia^{2,3} and fishes^{4,5} for example, little use seems to have been made of the urodele testis as a model for studies of this type. I present here a short account of spermatogenesis in the axolotl (*Ambystoma mexicanum*), which seems to be particularly suitable for this purpose. In addition to the more usual histological procedures, samples of the tissues were incubated with dehydroepiandrosterone (DHA) or Δ⁵-pregnenolone, in the presence of NAD as carrier and NBT as acceptor for hydrogen (without further staining) in order to demonstrate Δ⁵-3β-hydroxysteroid dehydrogenase (3β-HSDH) activity, according to the method described by Baillie *et al.*⁶.

As in the testis of other urodeles, the early stages of spermatogenesis in *A. mexicanum* occur within the tubules. With the occurrence of the first meiotic divisions, the original tubular form of the testis is replaced by cysts which exhibit a spermatogenic gradient from the periphery to the hilum. Within any one region, however, essentially all the cysts contain germ cells

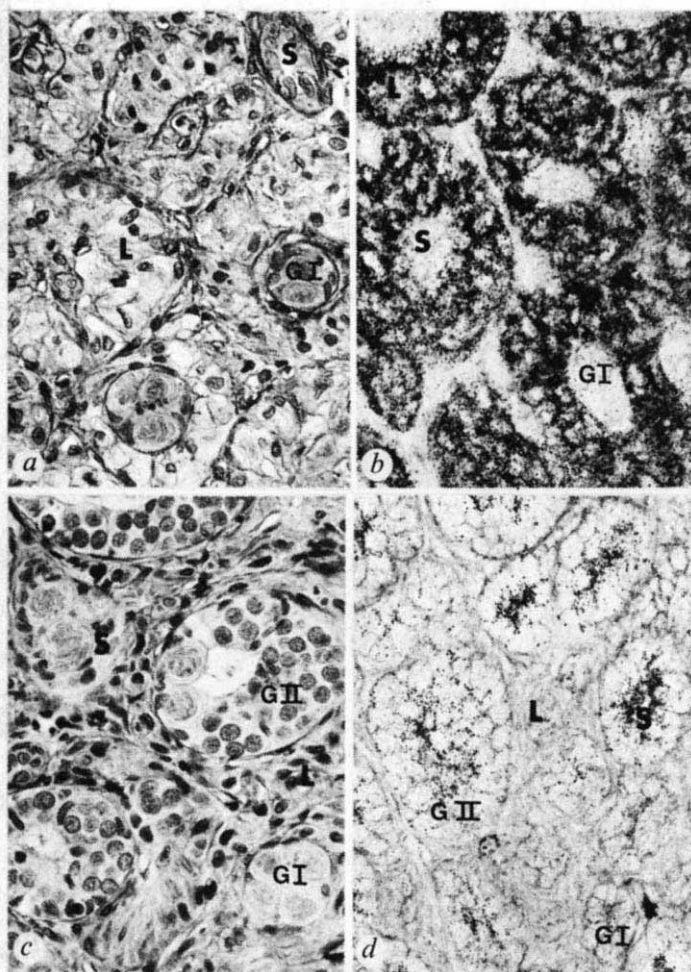


Fig. 1 Correlation between spermatogonial divisions and Δ^5 - 3β -HSDH activity in the glandular tissue. *a*, Interstitial tissue with enclosed nests of primary spermatogonia and Sertoli cells; *b*, demonstration of enzymatic activity restricted to the Leydig cells; *c*, Sertolien tubules with dividing germ cells, surrounded by regressing interstitial Leydig cells; *d*, demonstration of enzymatic activity restricted to the Sertoli cells. L, Leydig cell; S, Sertoli cells; GI, primary spermatogonia; GII, secondary spermatogonia. Magnification $\times 156$.

in the same stage of development. From the young spermatocyte to the late spermatid stage, both Sertoli cells and Leydig cells are relatively rare and the response to any test for 3β -HSDH activity is negative.

During the later stages of spermatogenesis, during spermateliosis, the amount of interstitial tissue begins to increase, a change accompanied by a positive reaction for 3β -HSDH activity. These changes continue until after discharge of the sperm, when large amounts of interstitial tissue envelop the collapsed cysts and there is a relatively massive positive reaction for 3β -HSDH activity. In this 'glandular tissue', characteristic of the urodele testis, small nests of primary spermatogonia accompanied by two or three Sertoli cells, are found between the masses of interstitial cells (Fig. 1 *a*). These nests can readily be identified in sections treated for 3β -HSDH activity, where they display a negative response in sharp contrast to that of the enclosing interstitial tissue (Fig. 1 *b*).

The Sertoli cells then begin to divide, a process accompanied by the first appearance of the next generation of tubules. As this occurs, the spermatogonia also begin to divide and the amount of interstitial tissue starts to decline (Fig. 1 *c*). By this time it is clear that two opposite events are taking place; the emergence of 3β -HSDH activity in the Sertoli cells and its decline in the regressing interstitial tissue. The response of the Sertoli cells to tests of 3β -HSDH activity increases up to the end of the spermatogonial divisions when it ceases (Fig. 1 *d*). The

Sertoli cells by then are masked by the newly formed primary spermatocytes and the surrounding interstitium has practically disappeared. The primary spermatocytes then enter the first meiotic division and the next cycle continues as described above.

It can be seen from these results that the structure and general course of events in the testis of *A. mexicanum* make it easier to correlate changes in the Sertoli cells and interstitium with definite stages of spermatogenesis than it is in mammals. Although the study of 3β -HSDH activity only produces a limited amount of information on steroid metabolism by the Sertoli cells and interstitium, it is also interesting to compare briefly the results obtained in the present work with current views about the role of the above cells in mammalian spermatogenesis.

As has been variously claimed for mammals, it seems that axolotl Sertoli cells are capable of steroid metabolism which can, in turn, be linked with spermatogenesis. In mammals, the ability of Sertoli cells to metabolise various substrates to androgens has been most closely linked with meiosis. In the axolotl, however, Sertoli cells 3β -HSDH activity is most closely associated with the spermatogonial divisions. Of particular interest in the axolotl, is that the periods of maximum enzyme activity in the Sertoli cells and the interstitium occur at different times, that is, they are essentially out of phase with each other, a situation not unlike that reported for androgen production by the two cell types in mammals during development⁷. So, although steroid metabolism by the Sertoli cells in the axolotl is seen to be at its height during the early stages of spermatogenesis, 3β -HSDH activity of the interstitium increases rapidly during spermateliosis, reaching a maximum after spermiation. In this animal therefore, the activity of the Leydig cells seems to be related chiefly to the release of the sperm and the development of the secondary sexual characteristics which occurs at this time.

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Rapid increase of foetal corticosteroids after prostaglandin E_2

MATURATION of the foetal hypothalamo-hypophyseal-adrenal axis appears to be involved in the initiation of parturition in several species^{1,2}, but the limiting determinant of this maturational process has not yet been identified. In sheep, an increase in the plasma cortisol concentration of the foetus has been observed before parturition^{3,4} but it has been demonstrated that this increase is not preceded by an elevation in plasma ACTH⁵. It has been suggested that there is a maturation of the foetal adrenal responsiveness to ACTH stimulation^{6,7} and perhaps activation of those enzymes specifically associated with cortisol production⁸. Recently we have shown that a primary prostaglandin, prostaglandin E_2 (PGE_2), is present in the plasma of the foetal lamb and that its concentration increases during late pregnancy⁹. Here we examine the possibility that prostaglandins may be an essential factor in the stimulation of corticosteroid production in the foetus and report a very rapid increase in foetal corticosteroids during PGE_2 infusion into the common carotid artery of foetal lambs.

Foetal catheters were implanted into four animals under

conditions of strict asepsis on days 109, 116, 119 and 120 of pregnancy⁹, and the first infusion was given 15, 10, 7 and 9 days later respectively. Prostaglandin E₂ (1.6 µg min⁻¹) was infused in five experiments on four foetuses between days 126 and 133 of pregnancy. Prostaglandin F_{2a} (1.6 µg min⁻¹) was infused in three experiments on three foetuses between days 124 and 130 of pregnancy. Infusions were performed on three of these foetuses with a cross-over experimental design allowing a four day recovery period, while the fourth received two infusions of prostaglandin E₂ with the same recovery period. Samples (3 ml) were collected from the femoral artery in three foetuses and from the external jugular vein in one foetus before, during and after the prostaglandin infusion. Foetal heart rate, foetal blood pressure and foetal blood gases were determined as described previously¹⁰. Corticosteroids were measured by radioimmunoassay¹¹ using an antibody prepared against cortisol 21-hemi succinate-BSA, which showed significant cross-reactivity with only corticosterone (10%) and deoxycorticosterone (5%). There was less than 1% cross reactivity with all other steroids tested. The solvent blank was generally less than 10 pg per tube and has not been deducted. The interassay coefficient of variation was about 12%.

Prostaglandins were measured by radioimmunoassay⁸, the mean concentrations of PGE before infusion was 355 ± 78 (s.e.m.) pg ml⁻¹ and during infusion of PGE₂, 2089 ± 841 pg ml⁻¹. The concentration of PGF before infusion was 401 ± 54 pg ml⁻¹ increasing to 772 ± 128 pg ml⁻¹ during infusion of PGF_{2a}.

There was a rapid increase in foetal plasma corticosteroids within 30 min of beginning an intrafetal infusion of PGE₂ (Fig. 1). The concentration of corticosteroids in samples of foetal plasma taken 45 min, 30 min and 1 min before infusion averaged 19–24 ng ml⁻¹ but increased ($P < 0.01$) to 61 and 62 ng ml⁻¹ at 30 and 60 min respectively. The corticosteroids remained raised throughout the subsequent hour of saline infusion, but had fallen to basal levels by the following day. In contrast there was no significant effect of PGF_{2a} infused into the same foetal lambs (Fig. 2). All four lambs were delivered prematurely between 134 and 142 days gestation. This may have been related to the raised cortisol levels resulting from the earlier infusions.

During the control period before infusion of PGE₂ the foetal PaO₂ was 18 ± 1 mm Hg, PaCO₂ 51 ± 2 mmHg and pH 7.32 ± 0.01. The corresponding mean values 30 and 60 min

Fig. 1 Foetal plasma corticosteroid response ($P < 0.01$; mean ± s.e.m.) to a 1 h infusion of PGE₂ (1.6 µg min⁻¹) at $t = 0$ (infusion period = shaded area) for four foetal lambs (gestational age 128 ± 1 d). Saline was infused at the same rate (9.4 ml h⁻¹) during the hour preceding and the hour following the prostaglandin infusion. An analysis of variance for repeated measurements in the same subject was completed for the corticosteroid response to prostaglandin infusion. Tukey Honestly Significant Difference procedure was utilised to test for significance between means when multiple comparisons were made.

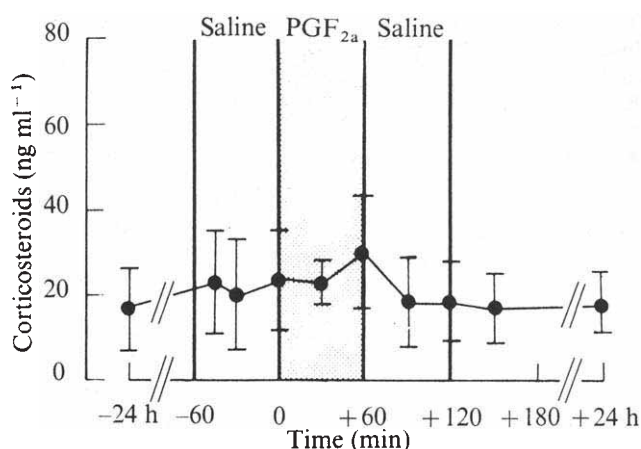
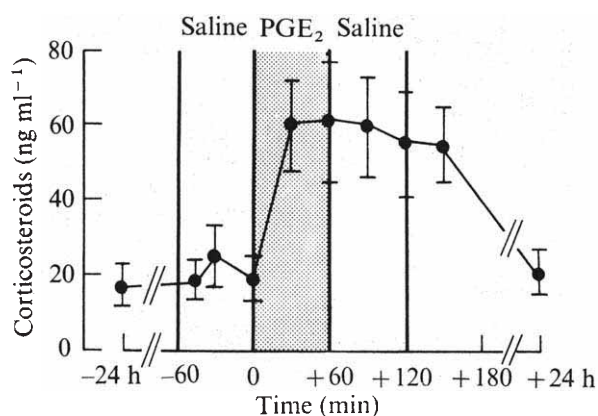


Fig. 2 Foetal plasma corticosteroid response (n.s.; mean ± s.e.m.) to a 1 h infusion of PGF_{2a} (1.6 µg min⁻¹) at $t = 0$ (infusion period = shaded area) in these same foetal lambs (127 ± 2 d). Prostaglandin infusions were administered in a 2 × 2 cross-over design with a 4-d recovery period allowed between experiments.

after beginning the infusion were PaO₂ 18 ± 0.0 and 17 ± 1 mmHg, PaCO₂ 52 ± 2 and 51 ± 2 mmHg and pH 7.32 ± 0.004 and 7.32 ± 0.010. The control values for the PGF_{2a} infusions were PaO₂ 21 ± 2 mmHg, PaCO₂ 50 ± 3 mmHg, pH 7.31 ± 0.0 and the values at 30 and 60 min after the start of the infusion were PaO₂ 20 ± 4 and 21 ± 3 mmHg, PaCO₂ 52 ± 1 and 50 ± 1 mmHg and pH 7.30 ± 0.01 and 7.31 ± 0.01. Foetal heart rate and blood pressure did not change during any of the infusions.

These studies have demonstrated that PGE₂, infused at a concentration which has no effect on any of the other physiological parameters measured, causes a rapid increase in foetal plasma corticosteroids. Our assay measures principally cortisol and other workers have shown that this is the major corticosteroid produced by the lamb foetal adrenal *in vivo*¹². Infusion of PGF_{2a}, on the other hand, does not alter the plasma corticosteroid concentration of the foetus. This could be due to the lower concentrations of PGF_{2a} achieved during the infusion but it does indicate that the stimulation observed with PGE₂ cannot be attributed to the experimental protocol or sampling regime we have used. The increase in foetal plasma corticosteroids caused by PGE₂ infusion is of importance because it occurred at a time when exogenous ACTH administered in large amounts over 1–2 hours (ref. 7), or endogenous ACTH, released in response to foetal hypoxia¹³ have little effect on foetal plasma corticosteroid concentrations. We have not measured ACTH in the present experiments, but in view of these previous studies, it seems unlikely that the prostaglandin effect is mediated by an increase in ACTH levels. Furthermore, it is unlikely that the present results are attributable to a transfer of maternal cortisol to the foetus, because in the sheep there is little transplacental passage of cortisol, even after raising the maternal plasma cortisol concentration considerably⁷. PGE₂ reduces umbilical blood flow¹⁴ and would not favour cortisol transfer to the foetus. Finally, the concentrations of PGE₂ achieved in the plasma in these experiments are similar to those we have reported at term⁹, thus raising the possibility that circulating PGE may at least augment any effect the trophic hormones may have on steroidogenesis in the foetal adrenal during late pregnancy.

Our results suggest that PGE₂ may exert a direct and specific action on the foetal adrenal gland. Our findings support those of Davies and Ryan¹⁵ who have found that the lamb foetal adrenal possesses those enzymes necessary for cortisol production by day 100–120 of pregnancy, despite the apparent insensitivity of the gland to ACTH at this time. *In vitro* studies with adult adrenal cells have suggested that prostaglandins may be involved in ACTH stimulated steroidogenesis^{13–15}.

The present results raise the possibility that the apparent insensitivity of the foetal adrenal gland to ACTH during much of pregnancy may be associated with an inadequacy of prostaglandin E production. However, our findings are also compatible with the idea that PGE₂ exerts its effect on the adrenal by stimulating the release of pituitary hormones, particularly since we made infusions into the carotid artery.

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Switch from foetal to adult haemoglobin synthesis in normal and hypophysectomised sheep

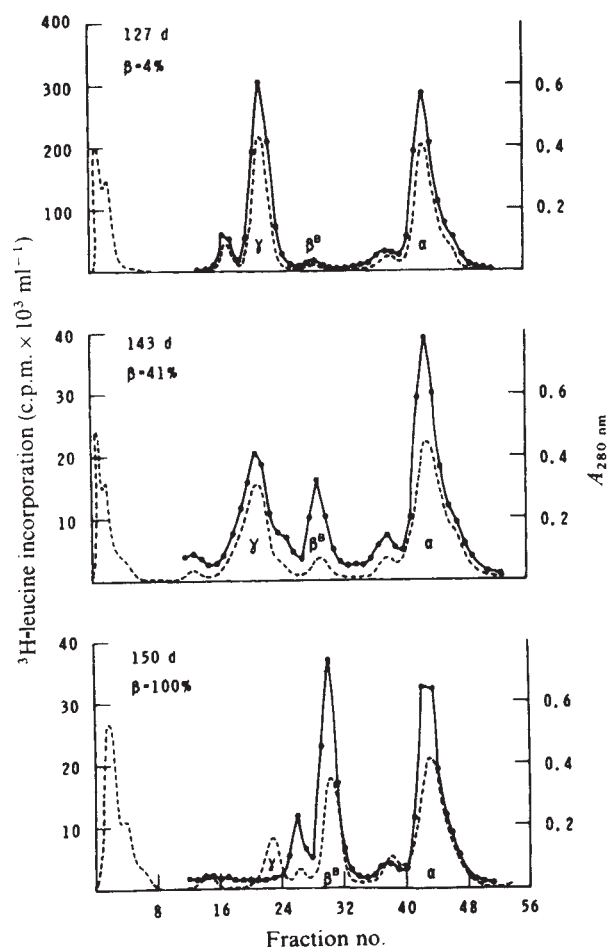
DURING the normal human perinatal period foetal haemoglobin (Hb F; $\alpha_2\gamma_2$) is replaced by the major and minor adult haemoglobins, Hbs A ($\alpha_2\beta_2$) and A₂ ($\alpha_2\delta_2$) respectively^{1,2}. Little is known about the mechanisms involved in the switch from γ - to β - and δ -chain production² but there is evidence that if the switch could be even partially prevented, so allowing continued production of γ chains into adult life, it might be possible to improve the outlook for patients with the common genetic disorders of β -chain production, particularly sickle-cell anaemia and β thalassaemia¹. Investigation of the control of human haemoglobin switching has been hampered by the lack of a suitable animal model since none of the small mammals commonly used in the laboratory have a true foetal haemoglobin³. However, the developmental changes in the haemoglobins of the sheep broadly mirror those observed in man; two embryonic haemoglobins are replaced early in gestation by a single foetal haemoglobin⁴ which in turn is replaced by the adult haemoglobins at about the time of birth^{5,6}. The two adult haemoglobins of the sheep, Hb A and Hb B, are determined by pairs of alleles for their respective β chains, β^A and β^B . We have examined the switch from foetal to

adult haemoglobin in the sheep to determine whether it is sufficiently similar to that in man to provide a suitable model for studying the control mechanisms involved in haemoglobin switching. In addition, we have carried out preliminary experiments to determine whether the switch may be under hormonal control.

The haemoglobin types of the sheep (Border Leicester $\times$ Suffolk cross) used in this investigation were either AB or BB. A carotid or femoral artery of the foetuses was catheterised between 108 and 124 d gestation⁷, and blood samples were obtained from this time up to birth at 139–144 d and from the newborn lambs for periods up to 1 month post partum. 5 ml blood samples were collected into heparinised syringes and the plasma was removed by centrifugation at 4 °C and stored at –15 °C before radioimmunoassay of cortisol and prolactin^{8,9}. The red cells were used for measurement of globin chain synthesis by incubation with ³H-leucine¹⁰, followed by the separation of the individual globin chains by carboxymethyl-cellulose chromatography¹¹ and determination of the radioactivity incorporated into each chain¹².

The pattern of the globin chain synthesis during development in a normal sheep foetus is shown in Fig. 1. The β

Fig. 1 Globin chain synthesis during development in a catheterised sheep foetus (delivered at 144 d gestation), illustrating the switch from γ - to β -chain synthesis. Red cells from the foetus or the newborn lamb were incubated with ³H-leucine for 1 h in the medium of Lingrel and Borsook¹⁰, in which the AB serum was replaced by saline. The cells were then washed three times with saline, lysed with an equal volume of distilled water and whole cell 'globin' precipitated with acid acetone, without further purification¹². The 'globin' was chromatographed on a carboxymethyl-cellulose column in an 8 M urea 2-mercaptoethanol buffer¹¹ and the chains separated with a 0.0045–0.025 M Na₂HPO₄ gradient (total volume 400 ml; flow rate 30 ml h⁻¹).



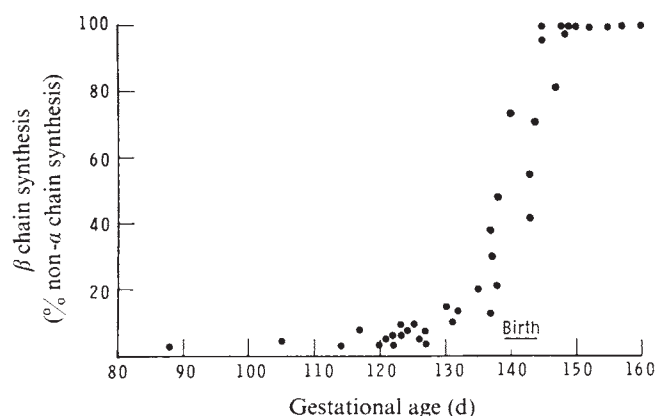
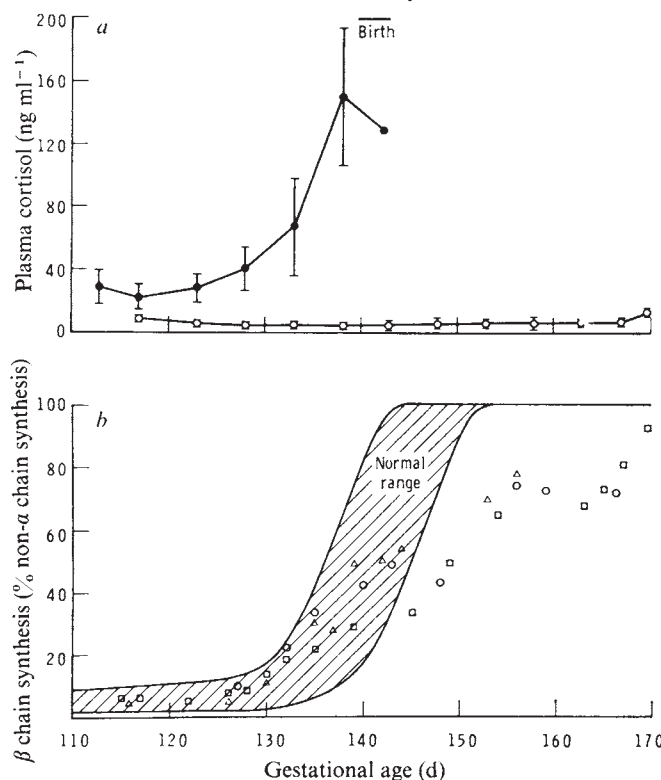


Fig. 2 β -chain synthesis, as a percentage of β - plus γ -chain synthesis, during development in eight catheterised sheep foetuses.

chains of adult Hb in this case were of the β^B type only and at 127 d gestation accounted for approximately 4% of the total non- α chains synthesised. By 143 d, however, the proportion had risen to 43% and by 150 d the switchover was complete and β -chain synthesis had totally replaced γ -chain synthesis. The combined data from eight normal foetuses are shown in Fig. 2. A low level of β -chain synthesis can be detected at early stages of gestation, as is the case in man¹³⁻¹⁵. The increase in β -chain synthesis is first seen at about 130 d gestation and replacement of γ -chain synthesis is virtually complete by 145–150 d. The whole process therefore takes only 2–3 weeks and is completed within a few days after birth, confirming a previous report¹ that the switch in sheep is considerably more rapid than that in man.

Although little is known about the control of the changes in haemoglobin synthesis during development, the involve-

Fig. 3 *a*, Mean plasma cortisol levels in seven normal (●) and three hypophysectomised (○) foetuses, $\pm$ standard error of the mean. *b*, Percentage β -chain synthesis in the three hypophysectomised foetuses compared with the normal range taken from Fig. 2. □, Hypophysectomised 114 d, delivered 173 d. △, Hypophysectomised 115 d, delivered 158 d. ○, Hypophysectomised 124 d, delivered 171 d. All hypophysectomised foetuses were delivered live but died shortly after birth.



ment of a humoral factor has been implicated by the findings of increased maternal Hb F production during early pregnancy^{16,17} and by the apparent synchrony of the switch in different erythropoietic organs¹⁵. For these reasons we have examined the effects of hypophysectomy on the relative rates of Hb F and Hb A production in the developing foetal lamb. Previous experiments have shown that in the absence of the pituitary, adrenal development does not occur, parturition is prevented, and the foetus continues to grow *in utero* past term¹⁸.

Three foetuses were hypophysectomised by coagulation diathermy¹⁸ at 114, 115 and 124 d gestation. The evidence that this was carried out successfully is based on the measurement of cortisol and prolactin levels in foetal plasma, as well as subsequent histological examination of the pituitary fossa. Plasma cortisol levels in the normal and hypophysectomised foetuses are shown in Fig. 3*a*. Clearly the sharp increase in cortisol levels which normally occurs a few days before birth has been abolished in the hypophysectomised foetuses, where the amounts remained at <20 ng ml⁻¹ until the pregnancy was terminated. Prolactin levels also remained low in the hypophysectomised foetuses as compared with the normals (I. C. McMillen *et al.*, unpublished). The birth weights of the three foetuses were 5.1 kg, 7.0 kg and 8.3 kg after 158, 171 and 173 d gestation respectively. The range for the normal newborn sheep at term was 3.5–6.5 kg. Measurements of packed cell volume and pO_2 in the hypophysectomised foetuses remained within normal limits throughout the post 'term' intrauterine period.

The pattern of the haemoglobin switch in the hypophysectomised foetuses is shown in Fig. 3*b*. An increase in β -chain production was observed at about the same time as that seen in the normal foetuses but the rate of increase was much reduced. The changeover to β -chain production, however, went almost to completion in two of the foetuses that survived to 170 d.

These studies suggest that the chronically catheterised sheep foetus provides an excellent model for examining the switch from foetal to adult haemoglobin production. The data from the eight normal foetuses examined here indicate that the switch is confined to a short period prior to birth and, once activated, proceeds extremely rapidly. In hypophysectomised animals the switch appears to begin at the same time as in normal animals but then proceeds more slowly. This suggests that the initial event in increasing β -chain synthesis may be independent of an intact pituitary but that the process is accelerated in its presence. Previous work (reviewed in ref. 19) has demonstrated the importance of the pituitary-adrenal axis and the production of cortisol in preparing the foetal lamb for extrauterine life. Clearly this preparation includes the replacement of foetal haemoglobin by the adult form but the precise role of the pituitary-adrenal axis remains to be determined.

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Changes in phosphorylation of P light chain of myosin in perfused rabbit heart

It is now well established that myosins from vertebrate skeletal and smooth muscles possess a light chain component of 18,000 to 20,000 molecular weight, the P light chain, that can be phosphorylated by an enzyme present in sarcoplasm^{1–3}. This enzyme, myosin light chain kinase, of molecular weight about 77,000 (ref. 4 and E. V. Pires and S.V.P., unpublished) is highly specific for the transfer of the γ phosphate of ATP to a single serine residue¹ close to the N terminus of the P light chain⁵. Fully phosphorylated myosin therefore contains 2 mol covalently bound P mol⁻¹ as two P light chains are present in each myosin molecule. Dephosphorylation of the P light chain is catalysed by an enzyme, myosin light chain phosphatase, that has recently been characterised⁶ and shown to be highly specific for the P light chain. Myosin light chain phosphatase has a molecular weight of 70,000 and is also present in the sarcoplasm. The presence of such a highly specific enzyme system for the phosphorylation and dephosphorylation of the P light chain strongly suggests a role in the physiological function of muscle. The amounts of the two enzymes present in skeletal muscle are such that it is unlikely that a complete cycle of phosphorylation and dephosphorylation of the P light chain can occur during a single twitch^{3,7}; it could, however, take place within a matter of seconds. We report here that changes in the phosphorylation of the P light chain in the perfused rabbit heart can be correlated with changes in the physiological state of the muscle.

Attempts to demonstrate differences in the enzymic

behaviour of myosin as a consequence of phosphorylation have been unsuccessful with the protein isolated from white skeletal muscle⁸, although there are reports that the low level of ATPase activity of actomyosin from smooth muscle can be increased by phosphorylation of the P light chain⁹. A possible indication of the role of phosphorylation of the P light chain is given by comparison of the myosin light chain kinase and phosphatase activities of different types of rabbit muscle³. Whereas the phosphatase activity is similar per gram wet weight in all muscle types so far studied, the kinase activities are much greater in skeletal than in cardiac and smooth muscles. The enzymic data suggest that if both enzymes are fully active in fast skeletal muscle the P light chain would be in the phosphorylated rather than the dephosphorylated state. On the other hand, in smooth and cardiac muscle, where the enzymic levels are more evenly balanced, changes in phosphorylation of the P light chain might be more readily detected in physiological conditions. We have, therefore, investigated changes in the state of phosphorylation of the P light chain in the perfused rabbit heart. These studies are complementary to those reported on the phosphorylation of troponin I⁹ and in most cases, the analyses of the P light chains were carried out on the extracts obtained after the isolation of troponin I from the hearts used in this study⁹. When the light chain fraction of myosin alone was isolated the methods used for perfusion, measurement of force and homogenisation of the hearts were identical with those described in detail elsewhere (ref. 9 and R.J.S., S.V.P. and C. M. Roberts, unpublished). In these cases the affinity chromatographic step for the isolation of troponin I was omitted.

When the hearts were removed from rabbits under anaesthesia by freezing *in situ* with a Wollenberg clamp the myosin was fully phosphorylated. The P light chains of myosin isolated immediately after death from the hearts of rabbits killed by stunning were only partially phosphorylated. If, however, the hearts from animals killed by stunning were perfused for 10–15 min with modified Krebs Henseleit buffer (ref. 9 and R.J.S., S.V.P. and C. M. Roberts, unpublished) the phosphate content of the myosin returned to 2 mol mol⁻¹; that is, the light chain fraction was fully phosphorylated (Table 1).

Adrenaline was added to the perfusion medium in the range 0.2–4.0 μ M and the light chains isolated from the homogenate prepared when the increase in force was maximal (usually 20–30 s after adrenaline addition) as described in the experiments in which the effect of adrenaline on the level of phosphorylation of troponin I was studied⁹. The phosphate content of the P light chain fell during the inotropic response, but although the

Table 1 Effect of procedure used for isolation of the rabbit heart on the level of phosphorylation of the P light chain of cardiac myosin

Procedure	No. of determinations	P content of myosin (mol mol ⁻¹)*
Excised and homogenised after death by stunning	5	1.0 $\pm$ 0.28
Anaesthetised with nembutal, heart homogenised after freezing <i>in situ</i>	3	2.2 $\pm$ 0.20
Killed by stunning: perfused 15 min	7	2.0 $\pm$ 0.19
Killed by stunning: perfused 100 min	3	2.1 $\pm$ 0.07

Ventricles were homogenised in 8 M urea, 75 mM Tris, 45 mM HCl, 1 mM CaCl₂, 15 mM 2-mercaptoethanol, pH 8.0 (ref. 9) and troponin I was removed by affinity chromatography^{9,10}. The bulk of the protein in the eluate was precipitated by an equal volume of ethanol and a crude light chain fraction was precipitated from the supernate by 10% (w/v) trichloroacetic acid. The precipitate was dissolved in 0.5 ml 1.0 M NaOH, diluted with 25 ml of 4 M urea, 50 mM Tris, 30 mM HCl, 15 mM 2-mercaptoethanol, pH 8.0, applied to a DEAE cellulose column and washed in with the same buffer containing 32 mM KCl. The cardiac light chains were eluted with the buffer containing 83 mM KCl, precipitated with 10% trichloroacetic acid and washed with 5% trichloroacetic acid before analysis for nitrogen and phosphorus¹¹. The proportion of the total protein in the fraction represented by the P light chain was estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis at pH 7.0; (ref. 1) relative amounts of protein in each band were determined for each estimation by a dye elution technique¹².

* Assuming 2 mol P light chain per mol myosin²; $\pm$ standard error of mean.

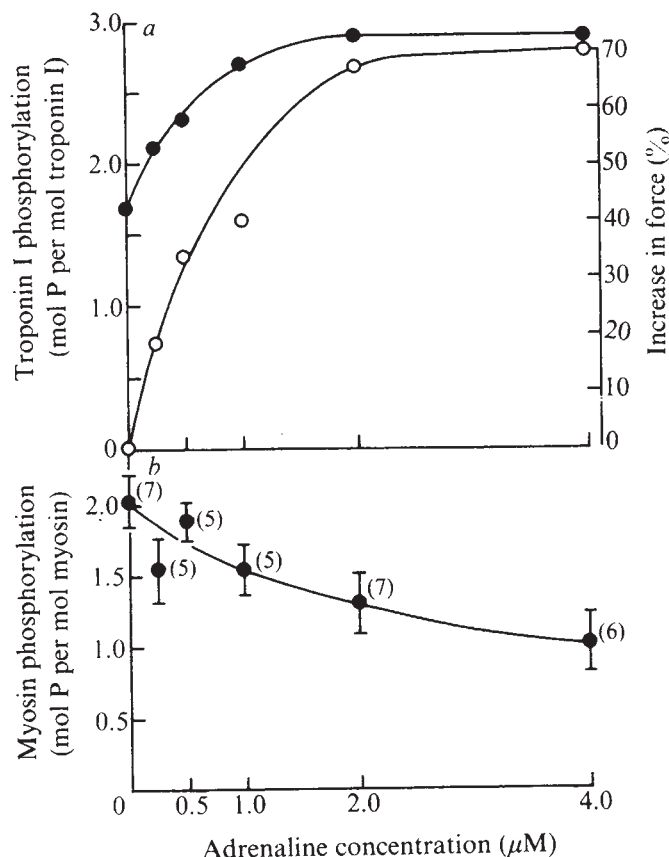


Fig. 1 Phosphorylation of the P light chain of myosin and of troponin I associated with the inotropic response of the perfused rabbit heart to adrenaline. See text for experimental conditions. Number of experiments carried out indicated in parenthesis. *a*, Changes in phosphorylation of troponin I and the increase in force developed on addition of adrenaline to the perfusate. From Solaro *et al.*^{9,11}. *b*, Changes in phosphorylation of the P light chain of myosin on addition of adrenaline. Most determinations were carried out on the same extracts as used in the experiments illustrated in *a*.

maximum contractile response was obtained at 2 μM adrenaline, the phosphate content continued to fall with increasing adrenaline concentration reaching 1.1 ± 0.20 mol per mol of myosin at 4 μM adrenaline, that is, about 50% of the control value (Fig. 1). If perfusion was continued for 5–10 min after the inotropic response, by which time force had returned to the level obtained before adrenaline addition, the phosphate content of the P light chain fraction returned to the original value, 2.0 mol per mol of myosin.

A similar increase in contractile force to that obtained with 2–4 μM adrenaline could be obtained by reducing the Na^+ concentration from 144 mM to 55 mM and increasing the Ca^{2+} concentration of the perfusion medium from the normal value of 2.5 mM to 7.5 mM. When the light chain fraction was isolated at maximum response as in the previous experiments the phosphate content fell to a value equivalent to 0.8 ± 0.07 mol per mol of myosin. Under these conditions there was no change in the troponin I bound phosphate which remained at the control value of 1.6 mol mol^{-1} (ref. 9).

When Ca^{2+} was excluded from the perfusion medium the hearts stopped beating after 30 s at which moment the phosphate content of the P light chain corresponded to 1.3 ± 0.13 mol per mol myosin.

The findings reported represent the first demonstration of a change in the state of phosphorylation of the P light chain of myosin that apparently correlates with a change

in physiological response of muscle. In both cases in which the force developed by the myocardium increased, dephosphorylation of the P light chain occurred.

The two forms of intervention employed to increase force production in the myocardium, that is, increasing Ca^{2+} and lowering Na^{2+} concentrations, and adding adrenaline to the perfusate would both be expected to increase the Ca^{2+} concentration within the cell. Such a change in internal Ca^{2+} concentration should favour phosphorylation of the P light chain for myosin light chain kinase requires this cation for activity⁴. The results reported indicate that the reverse is the case as net dephosphorylation of the P light chain takes place under both conditions. In contrast, on adrenaline treatment the activation of 3':5'-cyclic AMP-dependent protein kinase leads to the expected increased phosphorylation of troponin I⁸. Thus when the myocardium responds to adrenaline by increased force production, net phosphorylation and dephosphorylation occur simultaneously at specific sites on the contractile and regulatory proteins of the functioning myofibril. If the contractile force is raised by decreasing the Na^{2+} and increasing the Ca^{2+} concentrations in the perfusion medium, however, only the phosphorylation level of the P light chain appears to change.

Without further evidence it would be presumptive to conclude at this stage that dephosphorylation of the P light chain is directly related to the increased force developed by the myocardium. Nevertheless evidence suggesting involvement of the P light chain of myosin in the interaction with actin is beginning to accumulate and phosphorylation of this component might be expected to modify that interaction⁶. The analytical data available so far suggest that under the conditions studied, the increase in force in the myocardium is accompanied by a change in phosphorylation of the myosin from 2 to 1 mol per mol of myosin. If further investigation confirms that this is the case, the data could be interpreted to imply that only one of the light chains of each myosin molecule is dephosphorylated. Such an interpretation is intriguing and raises the question of the role of the two heads of the myosin molecule, in which the P light chains are located, and the function of phosphorylation in modulating possible cooperative effects associated with the heads.

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Directed selective pressure on a β -lactamase to analyse molecular changes involved in development of enzyme function

THE study of protein mutants has proved a powerful technique in the elucidation of structure-function relationships (see for example refs 1 and 2), but this approach has hardly been used strategically in the investigation of enzyme mechanisms, though its potential for clarifying the role of individual amino acids and their effect on catalytic rates is obvious. We report here the consequences of directed selective pressure on the plasmid-coded β -lactamase from *Escherichia coli* RTEM. Selection for strains with increased resistance to cephalosporin C has allowed the isolation of mutant enzymes whose catalytic properties are changed in the desired direction.

To trace the evolutionary improvement of an enzyme in molecular terms and to watch the stepwise development of its catalytic apparatus, we need an enzyme susceptible to directed selective pressure towards an altered catalytic function. So that mutational changes may be fully interpretable, the tertiary structure must be known. These criteria are fulfilled by the plasmid β -lactamase from *E. coli* RTEM. The enzyme is a monomer (molecular weight approximately 27,000), its sequence is almost complete (R. Ambler, private communication), its crystal structure has been solved to 5.5 Å (ref. 3) and work at higher resolution promises well (J. R. Knox, private communication). Although the directed selection of mutant bacterial enzymes has been achieved previously⁴⁻⁸ and mutant β -lactamases with altered substrate activity spectra have been observed⁹; in no case, unfortunately, is the tertiary structure of the relevant enzyme available, and mechanistic interpretation of the observed amino acid changes has not been possible.

Since the β -lactamase from *E. coli* RTEM hydrolyses benzylpenicillin 30 times faster than cephalosporin C, and since cephalosporin C has reasonable antibiotic activity against Gram-negative bacteria, mutagenesis was followed by selection for strains with increased resistance to cephalosporin C. (Superficially, any extension of the range of antibiotic resistance encoded by a bacterial plasmid could create a biohazard. However, such species already exist, and cephalosporin C-resistant strains (for example, of *Haemophilus*) have been isolated in the clinical laboratory. At least 10% of plasmid-coded resistant enteric species

harbour a plasmid which produces a lactamase active on cephalosporin C, and it is evident that the current experiments will not produce anything that does not already have its counterpart in nature (S. Falkow, private communication; see also ref. 10). Indeed, the studies initiated may usefully contribute to our understanding of the basis of such plasmid-mediated resistance.) Because not all resistant colonies will have a more effective β -lactamase (some colonies may survive by lowering the accessibility of the antibiotic to the killing loci, and others may contain mutations in the killing loci proteins themselves), a second selection was devised to eliminate all colonies whose survival was not derived from a mutation in the plasmid. The plasmids from 415 mutant survivors were transferred to a cephalosporin C-sensitive nalidixic acid-resistant female strain, which was then challenged with cephalosporin C and the donor males eliminated with nalidixic acid. To ensure that all mutated plasmids were screened, transfer was done on plates. (Since plasmids are derepressed for further transfer after initial mating¹¹, transfer in broth immediately after mutagenesis could lead to the first-transferred plasmids "taking over" the culture.) The efficiency of plasmid transfer was 100%. In this way only those colonies containing a favourably mutated plasmid were isolated.

Of the lysates from cultures of the 77 colonies that survived the second selection, four strains had the same β -lactamase specificity as the wild type but appeared to contain much higher levels of enzyme, one strain contained a β -lactamase of altered substrate specificity, and one strain showed changes in both activity and substrate specificity (see Table 1). Lysates from the remaining cultures showed no significant differences from the wild type, indicating a facile mechanism for the emergence of plasmid-borne resistance to cephalosporin C that does not involve the β -lactamase. One obvious possibility is that the mutated plasmid codes for a protein that lowers cell permeability to the antibiotic¹².

To distinguish more precisely between strains that produce more of the wild-type enzyme and strains that produce enzyme with a higher k_{cat} , the crude lysates were titrated with rabbit anti-(wild type)- β -lactamase antibody¹³. This showed that strain h2, for instance, produces 20-30 times more enzyme of unchanged specificity (Table 1). Strains h3, h4 and h6 behave similarly. Mutagenesis evidently most readily leads to super-producers of wild-type β -lactamase. In contrast, strain h1 produces 11-18 times more β -lactamase than the wild type, but this enzyme has altered V_{max} values (Table 1). The purified h1 enzyme and

Table 1 β -lactamase activity of mutant strains of *E. coli* RTEM*

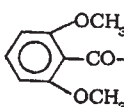
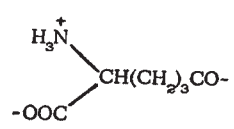
Mutant	Mutagen	Activity in crude extract†		Relative V_{max} ‡	
		Benzylpenicillin	Cephalosporin C	Benzylpenicillin	Cephalosporin C
h1	NTG	5.6	22	0.3	2
h2	EMS	22	31	1	1
h3	EMS	10	10	0.8	—
h4	EMS	10	14	0.6	—
h5	EMS	0.4	2.5	0.5	2
h6	NTG	2.5	2.5	1	1

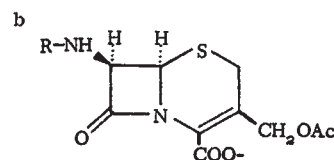
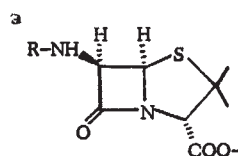
**E. coli* W3310 containing the plasmid RTEM was provided by Dr K. Sargeant of the Microbiological Research Establishment, Porton. Cultures were grown in 1% CY medium¹⁶. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was done¹⁵ at 100 μ g ml⁻¹ for 30 min. Mutagenesis with ethyl methane sulphonate (EMS) was done at 20 μ l ml⁻¹ for 90 min. Challenge with cephalosporin C was done on agar plates with antibiotic concentrations of 0.75, 1, 2, and 3 mg ml⁻¹. Surviving colonies after replica plating (to eliminate colonies surviving by virtue of antibiotic depletion) were transferred to a lawn of female *E. coli* C600, Leu⁻, Thr⁻, NaI^R (provided by Professor F. Ausubel), incubated for 90 min, then covered with soft agar containing cephalosporin C (400 μ g ml⁻¹) and nalidixic acid (20 μ g ml⁻¹). Survivors were purified twice on plates containing cephalosporin C (200 μ g ml⁻¹) and nalidixic acid (20 μ g ml⁻¹).

†Relative to a crude extract of the wild-type strain grown under identical conditions. The supernatant after lysis in the French press was used. Microiodimetric^{17,18} and ultraviolet¹⁹ assays were used.

‡Relative to the wild-type strain. Derived from the gradient of the antibody titrations, in which increasing amounts of rabbit anti-(wild type)- β -lactamase antiserum were added to a fixed number of units of β -lactamase activity. Residual enzyme activity was assayed after 15 min.

Table 2 Relative $V_{\max}$ values for penicillin (I) and cephalosporin (II) analogues with the purified enzyme from the wild type (WT) and from mutant h1

R:	H—	$\psi\text{CH}_2\text{CO—}$	$\psi\text{CH}(\text{NH}_3^+)\text{CO—}$		
penicillin analogue: $\frac{V_{\max}^{\text{h1}}}{V_{\max}^{\text{WT}}} (x)$	0.12	0.3	0.3	0.1	—
cephalosporin analogue: $\frac{V_{\max}^{\text{h1}}}{V_{\max}^{\text{WT}}} (y)$	11	0.75	0.16	3.5	1.4
specificity change (y/x)	90	2.5	0.5	35	—



the wild-type enzyme¹⁴ were both homogeneous on gel electrophoresis and isoelectric focusing, with no difference in the pI values, and no major changes in the tryptic peptide maps. Yet h1 is undoubtedly a true mutant (the LD_{50} with cephalosporin C for the wild type is $5 \mu\text{g ml}^{-1}$, and for h1 is $95 \mu\text{g ml}^{-1}$), and the h1 β -lactamase has catalytic properties altered in the expected direction (see Table 2). Strain h1 is a double mutant, producing more of a modified enzyme. (Double mutants are common when NTG is used¹⁵.)

From Table 2, it is evident that the h1 β -lactamase is, in $V_{\max}$ terms, 1.4-fold more effective in hydrolysing cephalosporin C (the K_m is also halved), and 3-fold less effective in the hydrolysis of benzylpenicillin (K_m unchanged). There is a more dramatic change when the unacylated β -lactam nuclei are compared: with 7-aminocephalosporanic acid, the h1 enzyme is more than 10 times more effective, yet with 6-aminopenicillanic acid the h1 enzyme is eight times less effective than the wild-type enzyme.

We have therefore achieved the desired alteration in the catalytic properties of the enzyme. The molecular basis of the changes in the catalytic effectiveness of this and other derived mutant enzymes is being investigated.

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Polypeptide elongation factor 1 and the control of elongation rate in rat liver *in vivo*

THE development of rapid kinetic methods for measurement of elongation rate in protein synthesis *in vivo*, independent of initiation rate, has made possible the study of the control of elongation in relation to changes in overall protein synthesis^{1–4}. In rat liver a 40% reduction in elongation rate (from about six to four amino acid residues per ribosome) is associated with thyroparathyroidectomy^{2,4}; the normal rate is restored by triiodothyronine injections². The work reported here concerns the role of polypeptide elongation factor 1 (EF1), the factor responsible for aminoacyl-tRNA binding to ribosomes, in the control of elongation rate in this system. Several studies have suggested that EF1 may have regulatory significance in eukaryotic systems^{5,6}; however no direct correlation between EF1 activity and elongation rate *in vivo* has previously been described.

Male Long-Evans rats, aged 2 months, were used in all experiments. Conditions for surgery and animal maintenance have been described⁷. Livers were quickly excised from decapitated animals and homogenised using a loose-fitting Dounce homogeniser in 3 vol of cold Medium A (0.25 M sucrose, 0.05 M Tris-Cl, pH 7.5, 25 mM KCl, 5 mM MgCl_2 , 5 mM 2-mercaptoethanol). Post-ribosomal supernatant fractions (S-100) were obtained by centrifugation at 15,000g for 10 min and 100,000g for 90 min at 4 °C. Polyribosome fractions were obtained from the 15,000g supernatant fraction by sedimentation for 4 h at 240,000g through a layer of 2 M sucrose containing the other ingredients of Medium A. About 40% of liver tRNA was recovered in the 240,000g pellet. For direct assay of bound EF1, polyribosome pellets were gently resuspended in Medium A at 20–30 A_{260} units ml^{-1} . Contrary to another report⁸, no increase in EF1 activity was obtained on

Table 1 Elongation factor I activity in S-100 fractions from livers of normal, thyroidectomised, and triiodothyronine-treated rats

Group	Average body weight (g)	Growth rate (final 7 days) (g d ⁻¹)	Binding activity* (Units per mg protein)	Polymerisation activity† (Units per mg protein)
Normal	218	6.2 ± 1.4	127 ± 16 (26)	53 ± 4 (20)
Thyroidectomized	155	2.1 ± 2.3	126 ± 18 (14)	55 ± 5 (14)
T3-treated	187	n.d.	139 ± 17 (10)	n.d.

*1 unit = 1 pmol ³H-phenylalanyl-tRNA bound min⁻¹ at 30 °C in a system containing 30 mM Tris-HCl, pH 7.5, 120 mM NH₄Cl, 5 mM MgCl₂, 4 mM 2-mercaptoethanol, 25 µg poly(U) acid (Miles Laboratories), 0.4 mM GTP, 2 *A*₂₆₀ units puromycin-treated rat liver ribosomes, 18 pmol (16,000 c.p.m.) ³H-phenylalanyl-tRNA and 1–5 µg S-100 protein in a final volume of 0.12 ml. After reaction for 5 min, binding was assayed on nitrocellulose filters¹¹. Results are presented as mean ± s.e.d. (number of animals). Each preparation was assayed 3–5 times. Polymerisation activity was <10% on the basis of recovery of phenylalanine-containing peptides by paper chromatography¹⁵ and on filter paper disks⁹ washed in hot trichloroacetic acid. Protein was estimated by the method of Schaffner and Weissman¹⁶.

†1 unit = 1 pmol ³H-phenylalanyl-tRNA incorporated min⁻¹ at 30 °C in a system containing 50 mM Tris-HCl, pH 7.5, 80 mM NH₄Cl, 4 mM MgCl₂, 4 mM dithiothreitol, 25 µg poly(U), 0.4 mM GTP, 0.5 *A*₂₆₀ unit puromycin-treated rat liver ribosomes, 20 µg EF2 protein, 16 pmol ³H-phenylalanyl-tRNA and 2–10 µg S-100 protein in a final volume of 0.12 ml. Incorporation into polyphenylalanine after 0 min reaction was determined on filter paper disks⁹.

n.d., not determined.

substitution of 1% Triton X-100 or Brij-58 for 0.5% deoxycholate. Bound EF1 was released by treatment of polyribosomes with NH₄Cl at 0.5 M (salt washing). EF1-dependent binding of aminoacyl-tRNA to ribosomes was assayed in a polyuridylic acid system¹⁰, using rat liver ribosomes isolated by sedimentation at 100,000g for 2 h and treated with puromycin for release of endogenous mRNA and peptidyl-tRNA¹¹. Polymerisation of polyphenylalanine was measured in a similar system containing an excess of EF1-free EF2 prepared by Sepharose 4B chromatography¹⁰, or a saturating level of EF1 purified by CM-Sephadex chromatography¹² and free of EF2. Rat liver transfer RNA charged with ³H-L-phenylalanine (New England Nuclear) was prepared by standard procedures¹³.

Table 1 summarises the results of EF1 assay for S-100 fractions of liver from normal (including sham-operated) and thyroidectomised rats, as well as animals given excess hormone (40 µg per 100 g body weight) for 3 d before experiment. No significant differences in EF1 activity were obtained by either assay. Time and concentration dependencies in the assays also were identical for the three groups. This activity therefore does not correlate with the changes of elongation rate found in these animal groups *in vivo*^{2,4}. The result contrasts with that in temperature-acclimated toadfish where soluble EF1 levels and elongation rate in liver show coordinate changes during metabolic compensation for temperature change^{1,5,17}. The difference may be due to an excess of the binding activity in rat liver, about three times that of fish liver. Elongation rates for the two species are nearly identical when body temperature difference is taken into account (from *Q*₁₀ = 3.0)¹⁸. An apparent excess of binding component (EFTu) also has been reported for *E. coli*¹⁹.

In contrast to the results for soluble EF1, direct assay for ribosome-bound EF1 in polyribosome preparations revealed significantly lower activity from thyroidectomised rats compared with controls (Fig. 1a). Salt-wash fractions obtained by extraction of polyribosome pellets with 0.5 M NH₄Cl also showed an approximately twofold difference in EF1 activity (Fig. 1b). Results are expressed relative to absorbance units of polyribosomal RNA from which the EF1 activity was derived. No other differences between polyribosome preparations from control and thyroidectomised rats were noted. Polyribosome recovery in the 240,000g pellet was the same for the two groups (2.9 ± 0.4 mg RNA per g liver), as was the protein yield in the NH₄Cl extract (0.32 ± 0.10 mg protein per mg RNA). Polyribosome profiles from liver post-nuclear supernatants of a matched pair of animals were indistinguishable from those of unoperated controls, as presented elsewhere²¹. Levels of EF2 activity in the NH₄Cl extracts (Table 2) were identical for normal and thyroidectomised rats.

Table 2 summarises the results for ribosome-associated EF1 and EF2. An EF1-dependent polymerisation assay

was also performed on the salt-wash fractions, with results comparable to those of the binding assay. The data show a 60% reduction in salt-extracted ribosome-bound EF1 activity in the thyroidectomised animals; the range at 95% confidence is 40%–75%. The observed change in elongation rate *in vivo*^{2,4} falls within this range.

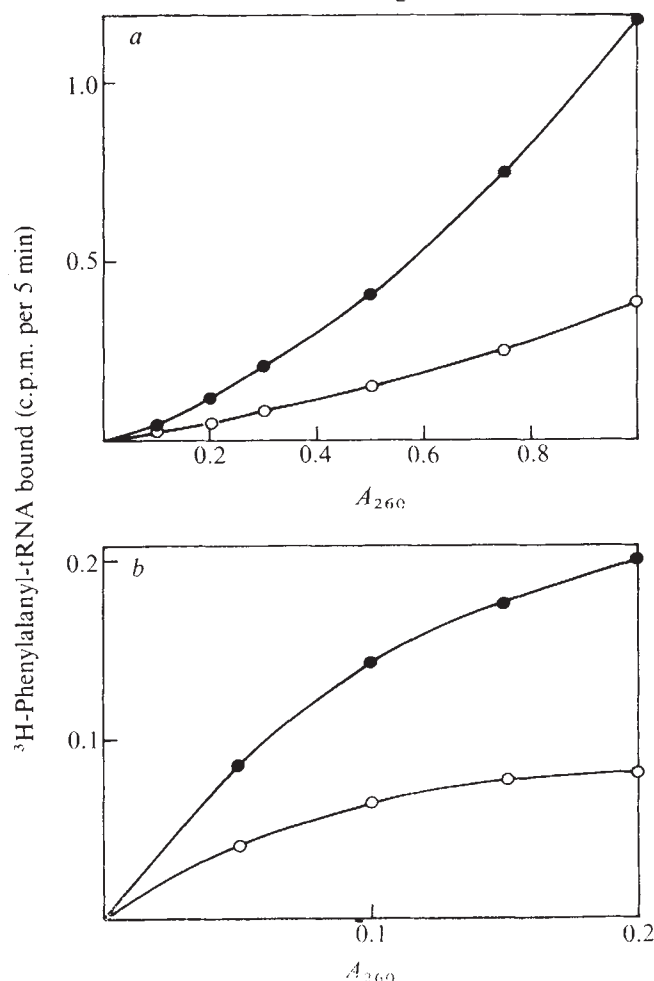


Fig. 1 *a*, EF1-dependent binding activity of polyribosome preparations of normal (●) and thyroidectomised (○) rat liver as a function of absorbance units at 260 nm of rRNA added to assay. *b*, EF1-dependent binding activity of supernatant fraction obtained by salt washing of polyribosomes as in (*a*), as a function of *A*₂₆₀ units of rRNA used in preparation. The salt-wash fraction was the supernatant layer (4 ml) recovered after the polyribosome suspension of 4 ml treated with 0.5 M NH₄Cl was centrifuged 1.5 h at 340,000g through a 9-ml layer containing 1.5 M sucrose, 0.5 M NH₄Cl and the other components of Medium A. The binding assay contained all the ingredients indicated in the legend of Table 1 with the exception of S-100 protein. Puromycin-treated ribosomes were also omitted in the direct assay of the polyribosome fraction.

Table 2 Elongation factor activity in polyribosomes

Group	Average body weight (g)	EF1 Binding Activity*		EF2 Activity† Salt-wash fraction (Units per mg rRNA)
		Polyribosomes (Units per mg rRNA)	Salt-wash fraction (Units per mg rRNA)	
Normal	186	8.0 ± 2.0 (14)	55 ± 14 (12)	40 ± 8 (12)
Thyroidectomised	133	3.2 ± 1.0 (15)	24 ± 8 (13)	45 ± 6 (13)

*Assay as described in Table 1 with the exception of S-100 protein. Polyribosomes were assayed in the range of 0.1-0.5 A_{260} unit in the final volume of 0.12 ml; 30% of the apparent binding activity was found to be associated with polymerised species. Salt-wash fractions were assayed in the range of 0.01-0.05 A_{260} unit of rRNA used in each preparation; these fractions gave less than 5% of the reaction product in polyphenylalanine. Data are presented as mean ± s.d. (number of animals). The preparation from each animal was assayed 3-5 times.

†EF2 was assayed in a polymerisation system as described in Table 1. In place of S-100 protein the system contained a saturating level ($5 \mu\text{g ml}^{-1}$) of purified rat liver EF1 and salt-wash fractions in the range of 0.02-0.1 A_{260} unit of rRNA used in preparation.

The molecular weight distribution of soluble and ribosome-bound EF1 activity from liver of normal and thyroidectomised rats was examined by sedimentation velocity in a sucrose gradient. As shown in Fig. 2a for a thyroidectomised rat preparation, EF1 activity in liver

S-100 occurs predominantly in small forms peaking at about 80,000 daltons with a leading edge extending to about 200,000 daltons. The distribution is consistent with the forms of EF1 previously identified in rat^{22,23} and pig liver¹². In contrast, EF1 activity obtained by NH_4Cl extraction of polyribosomes is about equally divided between small forms and aggregates in the region of 350,000-600,000 daltons (Fig. 2b). Control rats gave results identical to those of thyroidectomised rats.

The results above indicate that a component subject to assay in the polyribosome fraction may be the controlling factor in aminoacyl-tRNA binding and in polypeptide chain elongation in relation to thyroid hormone. It is apparently different from the major soluble binding activity, which does not change with hormonal status (Table 1). Some support for a qualitative difference in EF1 activity in the two compartments is provided by the sucrose gradient profiles (Fig. 2). Differences between soluble and ribosome-bound activity have also been reported for wheat embryo EF-1 (refs 24 and 25). The rate-limiting component could be a recycling factor, analogous to the bacterial elongation factor EFTs (ref. 26). Evidence for recycling of the activity associated with the binding component of pig liver EF-1 has been reported recently^{27,28}.

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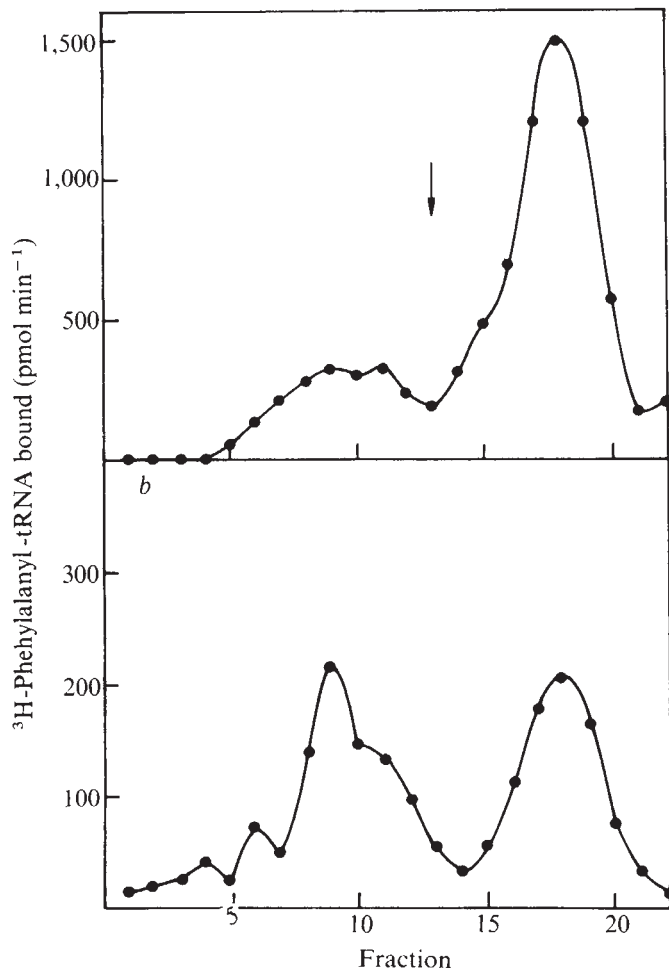


Fig. 2 Sucrose density gradient analysis of soluble and ribosome-bound liver elongation factor 1. *a*, EF1 binding activity in post-ribosomal supernatant (S-100). *b*, EF1 binding activity in the salt-wash fraction obtained from purified polyribosomes. Both preparations shown were obtained from a thyroidectomised rat. The S-100 was diluted 1:2.5 with 0.05 M Tris-HCl, pH 7.5, 2 mM 2-mercaptoethanol, and 50 μl was layered on a 5.2 ml 5-20% sucrose gradient containing the same buffer. The salt-wash supernatant fraction was chromatographed on Sephadex G25 equilibrated with the same buffer, and 200 μl was layered on the gradient. Centrifugation was carried out for 4 h at 62,500 r.p.m. in a Spinco SW65 rotor. Bovine serum albumin, human γ -globulin and catalase were run as markers; the position of catalase (11S) is indicated by an arrow in (*a*). Aliquot portions of 20 μl for (*a*) or 50 μl for (*b*) were assayed in the binding system described in Table 1. Sedimentation coefficients relative to marker positions were calculated by use of tables²⁹ and converted to molecular weights by an approximate equation $M = 6.6 \times 10^3 S^{3/2}$ based on the standard proteins.

Sodium ions and the shut-off of host cell protein synthesis by picornaviruses

TRANSLATIONAL control mechanisms have an important role in many cellular processes^{1,2}, but there is as yet little evidence showing the mechanism by which the control of protein synthesis at the translational level occurs. One of the best illustrated examples of translational control is observed when picornaviruses infect susceptible animal cells³. There is a rapid inhibition of cellular protein synthesis (the shut-off phenomenon) followed by a burst of viral protein synthesis, during which virtually no cellular proteins are made^{4,5}. Cellular mRNAs are not degraded with infection^{6,7}, but in spite of their presence the cellular protein-synthesising machinery only recognises and translates viral mRNAs.

In trying to formulate a model to explain the shut-off phenomenon the following experimental observations have to be accommodated: (1) the inhibition of host protein synthesis occurs at the initiation step⁸. (2) No replication of the virus is necessary for the shut-off to occur; under these conditions the shut-off depends upon the multiplicity of infection¹. (3) A viral protein factor is required; mutants defective in the ability to shut off map in a region of the genome that codes for the structural proteins⁹. (4) Cell-free protein synthesising systems obtained from uninfected cells

or cells infected with picornavirus are equally active in translating viral or host mRNAs *in vitro*^{10,11}. Together these data rule out the possibility of a specific modification of the protein-synthesising machinery after viral infection or the appearance of a stable macromolecular inhibitor such as double-stranded RNA^{12,13}, or a viral protein¹⁴. On the other hand the shut-off mechanism cannot be explained only by a direct competition between viral and cellular mRNAs¹⁵, because this is inconsistent with the observations mentioned above. Moreover, if cellular mRNAs were simply replaced by viral mRNAs this would not lead to the inhibition of total protein synthesis which is always found in infected cells.

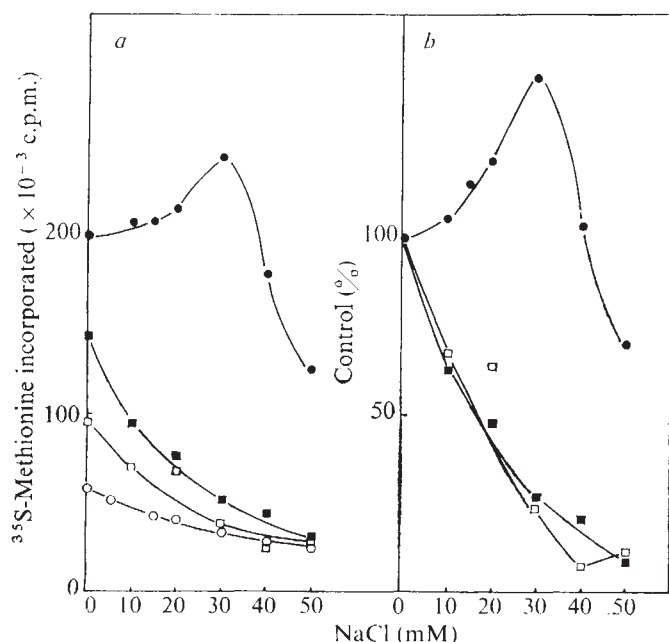
Increasing evidence has accumulated to show that after viral infection there are changes in the structure of cellular membranes^{16,17}. One likely consequence of this is a disruption of the ionic gradient between the inside and the outside of the cell. Indeed it has been shown that when *Escherichia coli* is infected by T7 bacteriophage, the membrane becomes leaky¹⁸, leading to a change of potassium concentration inside the cell¹⁹. The importance of such changes in monovalent cation concentration for cellular processes was pointed out by Lubin, when he observed that the rate of macromolecular synthesis depends absolutely upon the potassium concentration inside the cell²⁰. More recent experiments, notably those of Koch and his colleagues, also indicate the importance of monovalent ions in cellular processes; for example hypertonic medium causes the run off of HeLa cell polysomes²¹. Interestingly, if the cells are infected with poliovirus, viral protein is resistant to this inhibition²².

We here propose a model for shut-off which we believe takes into account all the observations mentioned. We suggest that following infection by picornaviruses there are changes in the cellular membrane which lead to an influx of sodium ions, and that this change in monovalent cation concentration results in the differential translation of host and viral mRNAs and leads to the shut-off of cellular protein synthesis. To test this we first examined the translation of different mRNAs *in vitro* in different salt conditions. Figure 1 shows that sodium ions cause an inhibition of protein synthesis directed by globin mRNA or by total mouse cell poly(A)-containing RNAs, in a cell-free system from ascites cells containing 80 mM KCl. A concentration of 30 mM NaCl inhibits protein synthesis directed by these mRNAs by about 80%. In the same cell-free conditions however, this concentration of sodium actually stimulates synthesis of the products directed by EMC RNA by almost 50%. A similar result was obtained with RNA from two other picornaviruses, mengovirus and poliovirus (unpublished observations). Polyacrylamide gel analysis of the proteins synthesised in the presence of sodium ions (Fig. 2) revealed an increase in the amount of the EMC-specific polypeptides and an inhibition of the synthesis of cellular proteins. Furthermore, the changes seen are only in the amount of protein synthesised, not its molecular weight.

The *in vitro* discrimination by sodium between viral and cellular protein synthesis occurs in various conditions. Figure 3 shows that the differences exist at all times during the incubation period and using different mRNA concentrations. In the control incubations containing no added sodium ions, approximately equal amounts of ³⁵S-methionine are incorporated in response to EMC RNA or cellular poly(A)-containing mRNAs, whereas, on addition of NaCl the translation of viral mRNA is favoured.

EMC RNA is efficiently translated *in vitro* over a wide range of potassium ion concentrations with a maximum at 110 mM KCl, whereas the translation of cellular mRNAs has a more stringent potassium ion requirement with a sharper optimum at about 80 mM KCl (Fig. 4). Addition of NaCl to the cell-free system shifts these optima such that in the presence of 30 mM NaCl maximal EMC RNA trans-

Fig. 1 Effect of sodium chloride on cellular and viral protein synthesis. Reaction mixtures contained (in 25 μ l): 20 mM HEPES, pH 7.0, 80 mM KCl, 1.5 mM MgCl₂, 6 mM 2-mercaptoethanol, 50 μ M spermine, 250 μ M spermidine, 200 μ M of each of the unlabelled L-amino acids minus methionine, 10–16 μ Ci of ³⁵S-methionine (specific activity 250–400 Ci mmol⁻¹), 1 mM ATP, 0.2 mM GTP, 10 μ l ascites S30, and where indicated 1 μ g EMC RNA, 1 μ g globin mRNA or 1 μ g mouse cell total poly(A)-containing mRNAs. The reaction was incubated for 90 min at 34 °C. At the end of this period 2 μ l of the reaction were withdrawn to analyse protein synthesis by trichloroacetic acid precipitation and subsequent filtration on nitrocellulose filters. The rest of the reaction was used to analyse the proteins on polyacrylamide gels as described²³. $\circ$, Endogenous protein synthesis; $\bullet$, plus EMC RNA; $\square$, plus globin mRNA; $\blacksquare$, plus mouse cell poly(A) containing RNA. *a*, Incorporation of ³⁵S-methionine at different sodium chloride concentrations; *b*, results expressed as a percentage of incorporation in the incubation containing no added sodium chloride.



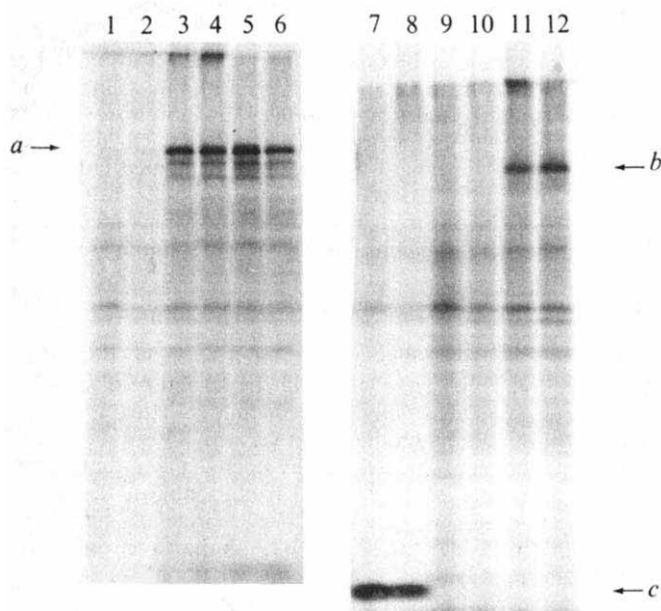


Fig. 2 Autoradiography of a polyacrylamide gel of the proteins synthesised *in vitro* under different conditions. 1, Endogenous protein synthesis under normal conditions (similar to those described in Fig. 1); 2, plus 30 mM NaCl; 3, plus 1 μ g EMC RNA; 4, 1 μ g EMC RNA, plus 15 mM NaCl; 5, 1 μ g EMC RNA, plus 30 mM NaCl; 6, 1 μ g EMC RNA, plus 45 mM NaCl; 7, 1 μ g globin mRNA; 8, 1 μ g globin mRNA : 30 mM NaCl; 9, 1 μ g cellular poly(A)-containing mRNAs; 10, 1 μ g cellular mRNAs plus 30 mM NaCl; 11, 2 μ g poliovirus RNA; 12, 2 μ g poliovirus RNA, plus 30 mM NaCl. A, B and C indicate the major products synthesised in response to EMC RNA, polio RNA and globin mRNAs respectively.

lation occurs at about 90 mM KCl. The KCl optimum for cellular mRNA also shifts on addition of NaCl, and using the conditions just mentioned (that is, 90 mM KCl + 30 mM NaCl), virtually no cellular protein is synthesised. Thus we conclude that it is the higher monovalent ion concentration in the incubation mixture which discriminates between the translation of EMC RNA and cellular mRNAs and not necessarily the presence of sodium ions. If under conditions of shut-off *in vivo* there were an ion influx, however, the physiological ion involved would be sodium.

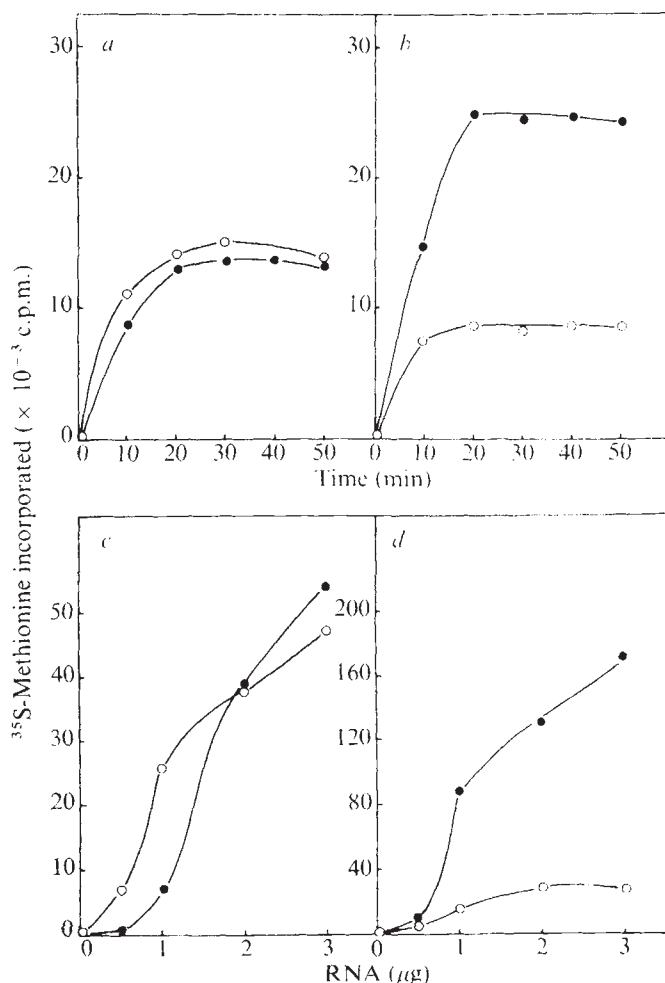
Earlier experiments have shown that the elongation rate *in vitro* is increased at higher potassium concentrations and consequently the product made in these conditions is of higher molecular weight²³; this raises the possibility that the stimulation by sodium shown here is merely a nonspecific effect on the elongation phase of protein synthesis *in vitro* which gives a differential response because EMC RNA is much longer than an average cellular mRNA. This is not the explanation in this case, however, because, as shown in Fig. 2, in our conditions the effect of higher salt concentration is quantitative and results in the synthesis of greater amounts of the protein coded for by the viral RNA but does not change its molecular weight. Thus this result is consistent with the view that there is more initiation on EMC RNA when sodium is present rather than more efficient elongation.

To examine the problem directly we have analysed the effect of sodium on the initiation of protein synthesis directed by globin mRNA or EMC RNA using the sparsomycin technique². Addition of sodium stimulated the formation of the EMC di- and tripeptides by up to 100% whereas the dipeptide formed with globin mRNA was decreased by about 60% (data not shown). We conclude that sodium inhibits the initiation of protein synthesis directed by globin mRNA and stimulates initiation directed by EMC RNA. At present we do not know what step in initiation is affected by sodium, but we believe that it probably has a

direct effect on the interaction between mRNA and ribosomes, thereby increasing the affinity of picornavirus RNA and decreasing the affinity of cellular mRNAs.

The experiments outlined above all indicate that sodium ions can discriminate between the translation of host and picornavirus mRNAs *in vitro*, and are consistent with our model for shut-off. A further prediction of our model is that, following infection, the intracellular concentration of sodium ions increases. Indirect evidence for this was obtained by measuring the activity of the enzyme responsible for the maintenance of the monovalent ion gradient (the Na^+/K^+ ATPase)²⁴ at different times after infection by estimating the uptake into cells of the potassium ion analogue $^{86}\text{Rb}^+$. In parallel experiments, viral protein synthesis was first observed 4 h post-infection and maximum synthesis was reached at 5–6 h post-infection (data not shown). Concomitant with the onset of viral protein synthesis the ability of the cell to take up $^{86}\text{Rb}^+$ becomes drastically impaired (Fig. 5). This indicates that either the Na^+/K^+ ATPase activity is severely inhibited or that the plasma membrane becomes leaky to monovalent ions. Either mechanism will result in the breakdown of the monovalent ion gradient, and lead to an increase in the sodium ion concentration inside the infected cell. Such evidence supports our hypo-

Fig. 3 Time course and RNA dependence of protein synthesis directed by EMC RNA or cellular poly(A)-containing mRNAs. Reaction mixture contained in 50 μ l the same components as indicated in Fig. 1 except that the specific activity of ^{35}S -methionine was 20 Ci mmol⁻¹. The potassium concentration was 80 mM. Endogenous protein synthesis was subtracted from each of the values given. ●, EMC RNA directed protein synthesis; ○, protein synthesis in response to total mouse cell poly(A)-containing mRNAs. a, c, Controls; b, d, + 30 mM NaCl.



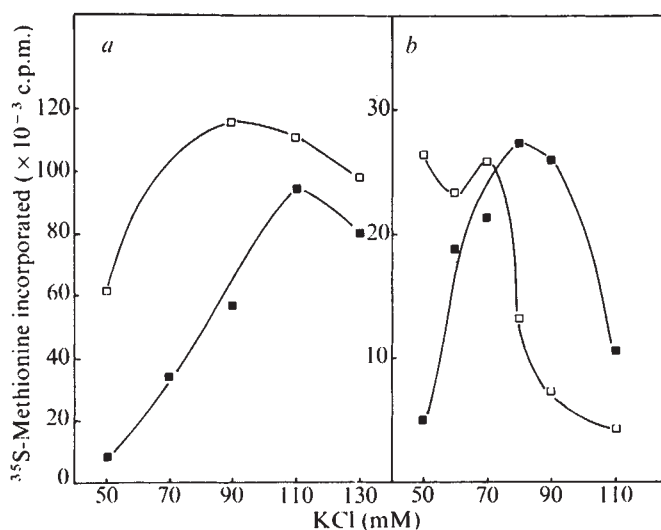


Fig. 4 Effect of potassium chloride concentration on protein synthesis in response to EMC RNA and cellular mRNAs in the presence and absence of sodium chloride. Conditions and components were basically as indicated in Fig. 2. *a*, Protein synthesis in response to EMC RNA and *b*, in response to total cellular poly(A) mRNAs. $\blacksquare$, No added sodium; $\square$, plus 30 mM NaCl. Endogenous incorporation was subtracted.

thesis that a redistribution of ions occurs following viral infection.

To summarise, we suggest that the shut-off phenomenon could be explained by supposing that after infection a viral protein is able to alter the gradient of monovalent ions maintained by the cell membrane, increasing the concentration of sodium in the cytoplasm and causing inhibition of

the initiation of cellular protein synthesis (polysomal run-off). In addition, the new ionic conditions in the cytoplasm would facilitate the translation of viral RNA because (1) there would be no competition from host mRNA and (2) the binding of viral RNA would be favoured by the increased monovalent ion concentration. Experiments examining the effects of sodium ions on the cell-free translation of host and viral mRNAs, and indirect measurements of the ionic gradient in virus-infected cells support the model.

The model presented here raises many questions. How does a viral protein change the ionic environment within the cell? Is this absolutely necessary for viral development? Does the change in ionic environment affect other cellular functions? Do all viruses use a similar strategy? It is equally possible that by the appropriate regulation of ion pumps, protein synthesis in uninfected cells could also be regulated by a similar mechanism since it is already known that cellular mRNAs also display a spectrum of resistance to excess salt.

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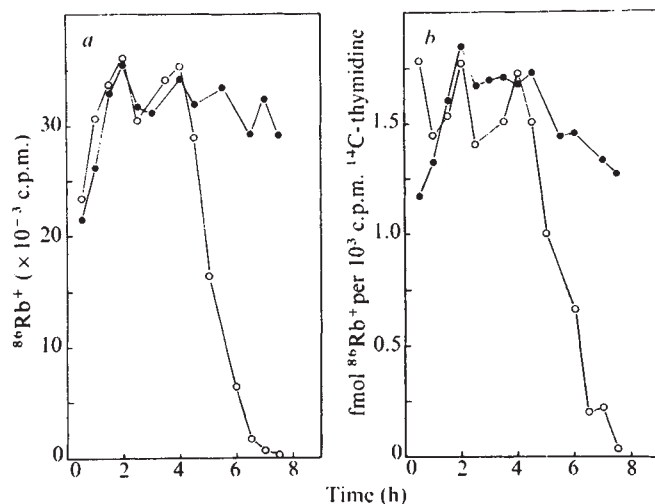
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Purification and characterisation of viral RNA of a sarcoma virus isolated from a woolly monkey

RNA-CONTAINING type-C viruses with the ability to induce morphological transformation of fibroblasts in cell culture have been isolated from both avian and mammalian species. In studies on such viruses, clonal isolates of the Rous sarcoma virus have been identified which are competent both for replication and for transformation¹. In contrast, in mammalian systems virtually all strains of sarcoma viruses thus far isolated are defective by themselves for replication². In order to obtain infectious sarcoma virus, helper type-C viruses must also be present to provide

Fig. 5 Mouse fibroblast 3T6 cells were grown in 3 cm diameter dishes containing 2 ml E4 medium with 10% calf serum and ^{14}C -thymidine (50 mCi mmol⁻¹, 0.05 $\mu\text{Ci ml}^{-1}$). When the cells were confluent, the medium was removed and the cells infected with encephalomyocarditis virus (20 PFU per cell). After 1 h at 37°C, 1 ml E4 medium containing 5% calf serum was added. The final concentration of KCl in the medium was 0.5 mM. Control or virus-infected cells were pulse labelled for 30 min at different times after infection with 1.25 $\mu\text{Ci ml}^{-1}$ $^{86}\text{Rb}^+$ (370 Ci mmol⁻¹). The medium was removed and the cell monolayer washed three times with phosphate buffer. 1.5 ml 5% trichloroacetic acid was added to fix the cells and the $^{86}\text{Rb}^+$, which leaks out from the cells, estimated by taking a 1 ml sample of the supernatant and measuring the Cerenkov radiation in a scintillation counter. The cell monolayer was washed several times with 5% TCA and twice with methanol. 0.2 ml of 0.02 N NaOH was added and a 0.150 ml sample was taken to estimate the ^{14}C -thymidine incorporated. *a*, Total c.p.m. of $^{86}\text{Rb}^+$ taken into the cells. *b*, Results of the $^{86}\text{Rb}^+$ uptake normalised to the ^{14}C -thymidine incorporated. ($\bullet$) $^{86}\text{Rb}^+$ uptake in control cells. ($\circ$), $^{86}\text{Rb}^+$ uptake in encephalomyocarditis virus-infected cells.



functions necessary for replication. To analyse the RNA subunits contained in heterologous type-C virus pseudotypes of defective sarcoma viruses, we have studied the RNA components obtained from a virus complex containing the woolly sarcoma virus and a mouse type-C helper virus. We have observed that the 45 to 70S high molecular weight RNA formed in this virus complex represents two distinct populations of high molecular weight RNA. One population is composed of subunits of mouse viral RNA, and the other population is composed of subunits of woolly sarcoma virus RNA. These two sets of apparent dimers³⁻⁵ have been readily separated from each other in sucrose gradients, thus providing a rapid, simple approach to obtaining large quantities of sarcoma virus RNA free of helper virus RNA. Using this approach, the nucleic acid sequences of the woolly sarcoma virus have been analysed.

In past experiments, the sarcoma virus first isolated from a spontaneous fibrosarcoma of a woolly monkey^{6,7}, was subsequently cloned in transformed nonproducer rat cells free of replicating woolly leukaemia virus^{8,9}. By infecting the non-producer cell⁸ with an amphotropic strain of mouse type-C virus¹⁰ we have produced a pseudotype containing the amphotropic mouse type-C helper virus and the woolly sarcoma virus. This complex was transmitted to BALB/c mouse cells and foci producing both viruses were cloned. One such clone of transformed cells, WB334, was analysed by RNA-³H-DNA hybridisation techniques for the relative viral RNA content of the mouse helper virus and the woolly sarcoma virus. To detect the mouse type-C viral RNA, a cDNA probe was prepared from the amphotropic virus grown in a strain of wild mouse cells, SC-1 (ref. 10). To detect the RNA content of the woolly sarcoma virus subunit, a cDNA probe from the woolly leukaemia virus was used since earlier experiments, hybridising Wo-1v ³H-cDNA to RNA of non-producer cells transformed by the Wo-sv, have indicated that the woolly sarcoma viral genome contained sequences homologous to the woolly leukaemia virus¹¹. A virus preparation from the WB334 clone was prepared and the RNA isolated in sucrose gradients as shown in Fig. 1.

Each fraction of the sucrose gradient was hybridised to the cDNA from the mouse type-C virus and to the cDNA from the woolly leukaemia virus. Two discrete peaks of hybridisation can be seen; under these stringent hybridisation conditions the faster sedimenting peak hybridises well to the cDNA homologous to the mouse type-C virus and the slower sedimenting peak hybridises to the cDNA from the woolly leukaemia virus. The peak tube of each of the two RNA peaks is separated by approximately five fractions in the sucrose gradient. In addition, there is no indication of high molecular weight RNA heteromers composed partly of mouse and partly of woolly RNA since the hybridisation profiles are almost completely non-overlapping. Thus, the results indicate that this pseudotype mixture contains two distinct populations of high molecular weight RNA. Assuming that the two-subunit model for the high molecular weight RNA of the tumour viral genome is correct³⁻⁵, then the faster sedimenting peak represents homodimers of amphotropic viral RNA and the slower sedimenting peak homodimers of woolly viral RNA. In experiments not shown, the high molecular weight form of each RNA was heated for 2 min at 80 °C and resedimented in sucrose gradients. After denaturation, the mouse viral RNA sedimented at 33–35S and the woolly sarcoma viral RNA at 24–26S.

In Fig. 1b, a large quantity of the virus mixture was disrupted with sodium dodecyl sulphate, and the RNA sedimented in sucrose gradients. The optical density at 260 nm was determined in each fraction. Two discrete peaks of absorbance can be seen similar to the results

obtained above in the hybridisation experiment shown in Fig. 1a.

The RNA in the slower sedimenting high molecular weight RNA was isolated from the sucrose gradient shown in Fig. 1b, and reverse transcribed *in vitro* with partially purified avian myeloblastosis virus reverse transcriptase¹¹. The ³H-cDNA probe prepared from the woolly sarcoma viral RNA was tested for its information content and the results were compared with a cDNA probe prepared from

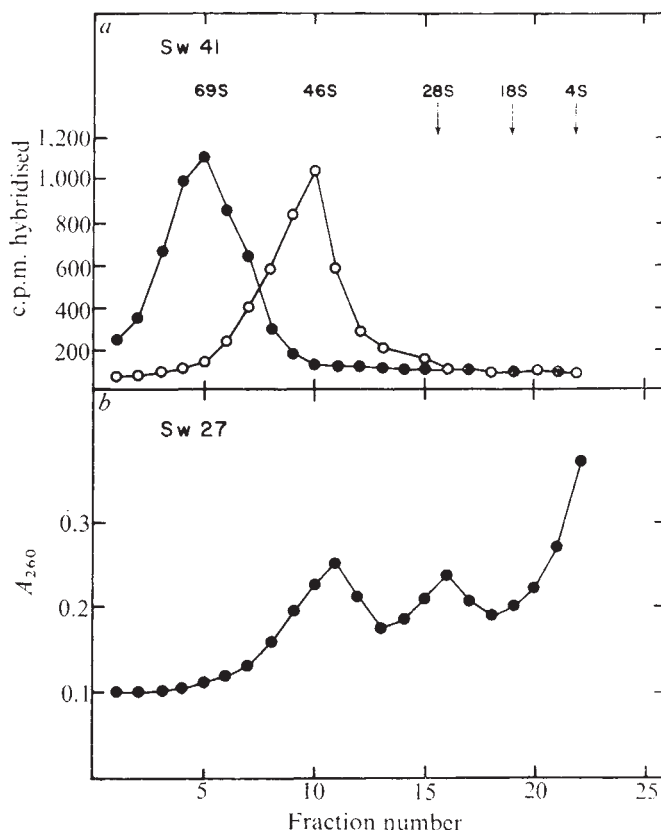


Fig. 1 Sucrose gradient analysis of viral RNA. *a*, Analytical gradient: 60 ml of supernatant fluid, representing a 6-h harvest of the culture fluid from the WB334 culture, were clarified at 5,000 r.p.m. at 4 °C for 10 min in a PR6000 centrifuge. The fluid was applied in 20 ml aliquots to a discontinuous sucrose gradient in an SW27 rotor tube containing: 4 ml of 60% sucrose and 12 ml of 30% sucrose prepared in 0.02 M Tris-HCl pH 7.5, 0.1 M NaCl, 0.001 M EDTA (TNE). The virus was centrifuged on to the 60% cushion at 25,000 r.p.m. at 4 °C for approximately 16 h. The virus was aspirated from the 60% cushion, diluted four-fold with TNE and centrifuged at 100,000g at 4 °C for 2 h concentrate the virus. The pellet was resuspended in 1.0 ml containing TNE and 1% sodium dodecyl sulphate. The disrupted virus was then centrifuged in a 15–30% sucrose gradient in TNE, in an SW41 rotor tube at 18 °C for 3.25 h at 39,000 r.p.m. 25 fractions of approximately 0.5 ml were collected by puncturing the tubes from below. 20 µg of yeast RNA were added to each fraction and the fractions precipitated with 2 volumes of ethanol. After collecting the ethanol precipitates, the fractions were resuspended in 100 µl of 0.02 M Tris-HCl pH 7.2 and 10 µl of each fraction was assayed for hybridisation as detailed below. Each hybridisation reaction contained ³H-cDNA (1 × 10⁵ c.p.m. µg⁻¹) either from an amphotropic mouse leukaemia virus (2,100 c.p.m.) or from the woolly leukaemia virus (2,500 c.p.m.). The amphotropic virus was grown in SC-1 cells, and the woolly virus in NC37 human cells¹¹. Hybridisation was performed and analysed with the use of sl nuclease by procedures fully described in earlier publications¹¹. *b*, Preparative gradient: 3 ml of the virus mixture produced from 25 l of the pseudotype virus mixture was disrupted with 1% sodium dodecyl sulphate and centrifuged for 4.5 h at 24,000 r.p.m. in an SW27 15–30% sucrose gradient at 18 °C. Fractions of 1.0 ml were collected by puncturing tubes from below, and the absorbance of each fraction at 260 nm was recorded. For the subsequent studies of the woolly sarcoma virus RNA, fractions 14 to 18 in Fig. 1b were pooled, and the RNA collected by precipitation with ethanol and centrifugation of the ethanol precipitate.

woolly leukaemia viral RNA. As shown in Table 1, the cDNA prepared from the woolly leukaemia virus RNA hybridises to both the woolly leukaemia virus and woolly sarcoma viral RNA; at saturation (C_{rt} of 1.0 mol l^{-1}) approximately 50% of the sequences contained in the woolly leukaemia cDNA are protected by the woolly sarcoma virus RNA. The results indicate, as previously suggested, that the woolly sarcoma virus contains only a portion of the woolly leukaemia genome¹¹.

The crude cDNA probe prepared from the woolly sarcoma viral RNA, which contains mostly woolly leukaemia virus sequences, was also hybridised to the same viral RNAs and showed slightly greater hybridisation to the woolly sarcoma virus RNA than to the woolly leukaemia virus RNA. After purification of the woolly sarcoma virus probe to remove woolly leukaemia virus sequences (see legend to Table 1), the woolly 'src' probe was re-tested for its specificity and the results are also shown in Table 1. The woolly src probe hybridised well to the woolly sarcoma virus RNA and not at all to the viral RNA from the woolly leukaemia virus, or viral RNA from RD114 virus, RT21c rat virus, or amphotropic mouse leukaemia virus. In addition, the woolly viral src sequences were detected in cellular RNA in non-producer rat cells transformed by the woolly sarcoma virus but not in rat cells transformed by the Schmidt-Ruppin strain of Rous sarcoma virus. These src sequences were also detected in horse cells producing both the woolly leukaemia and woolly sarcoma viruses but not in horse cells producing the woolly leukaemia virus alone. The results show that woolly sarcoma virus contains two sets of nucleic acid sequences. One set of the sequences is contained in the woolly leukaemia virus, and another set is specific for the woolly sarcoma virus. These results are consistent with finding on other avian and mammalian sarcoma viruses which showed their genomes to contain both leukaemia virus sequences and distinctive sequences specific to the sarcoma viruses^{2,12,13}. In order to quantitate the relative percentage of the two sets of sequences in the woolly sarcoma virus genome, ³²P radio-labelled RNA was prepared from the woolly sarcoma virus, by labelling the viruses

released from WB334 cells with inorganic ³²P and separating the high molecular weight RNAs as shown in Fig. 1b. The ³²P-labelled woolly sarcoma virus RNA was then hybridised to a two-fold molar excess of ³H-cDNA from the woolly leukaemia virus, or to a two-fold excess of cDNA purified to represent the novel sequences in the woolly sarcoma virus. At these ratios of DNA to RNA, the src probe protected 52% of the ³²P-labelled RNA and the Wo-lv cDNA protected 52% of the ³²P-labelled RNA.

Thus we have analysed the RNA subunits contained in the virus complex containing an amphotropic mouse type-C virus pseudotype of the woolly virus. The data indicate that the high molecular weight forms of the two viral RNAs in this virus complex are essentially completely separable by velocity sedimentation in sucrose gradients and that no detectable heterodimers occur between the mouse type-C viral RNA and the woolly sarcoma viral RNA. The woolly sarcoma viral RNA is smaller than the accompanying helper viral RNA, and contains only a portion of the Wo-lv genome. In the woolly sarcoma viral RNA a set of sequences has been detected which comprise approximately 15% of the woolly sarcoma genome; the sarcoma virus specific sequences are not detected in the woolly leukaemia virus. Thus, the genome of the naturally occurring Wo-sv seems to be similar to the genomes of rodent sarcoma viruses formed in laboratory experiments^{14,15}; that is the sarcoma virus genome represents a set of new sequences plus a portion of the genome of the helper virus contained in the original virus stock. The ability to obtain large quantities of purified RNA and cDNA specific for the woolly sarcoma virus should allow an identification both of the origin of the sarcoma-specific sequences of the woolly sarcoma virus and possibly by *in vitro* translation, identification of any possible gene product(s) of these sequences.

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Table 1 Hybridisation with woolly leukaemia and woolly sarcoma virus cDNAs

RNA Source	c.p.m. hybridised with ³ H-cDNA from	
	Wo-lv	Wo-sv
Viral		
Wo-lv	2,116	Crude 965 Purified 18
Wo-sv	1,158	1,150 33
RD-114	41	98 10
Amphotrope	186	156 25
RT21C	38	108 13
None	43	93 10
Cellular		
Non-producer cells		
Wo-sv NRK	—	— 333
SR NRK	—	— 22
Producer cells		
Wo-lv NRK	2,230	— 12
Wo-lv horse	2,165	— 12
Wo-lv/Wo-sv horse	2,177	— 308

Each hybridisation with woolly leukaemia virus ³H-cDNA contained 2,500 TCA c.p.m. The hybridisations with the crude woolly sarcoma virus cDNA contained 1,800 TCA c.p.m. and with the purified woolly sarcoma virus cDNA 630 TCA c.p.m. The sarcoma-specific cDNA was obtained by hybridising the crude cDNA to woolly leukaemia virus RNA and subsequent chromatography on hydroxylapatite^{14,15}. The hybridisations with viral RNA were carried out to C_{rt} values of 1.0 mol l^{-1} and with cellular RNA to C_{rt} values of $5 \times 10^9 \text{ mol l}^{-1}$. Analytical hybridisation conditions and analysis with the use of sl nuclease have also been fully detailed in earlier publications¹¹. The source of the cells has been described¹⁴ except for the horse cell which was obtained from the American Type Cell Collection.

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Errata

In the letter "Stab initiation of explosions" by M. M. Chaudhri (*Nature*, **263**, 121; 1976) the expression giving q_1 in equation (2) should be

$$q_1 = \left[1 + \sqrt{\left(\frac{\pi}{2} \frac{\nu(1 - \sin^2 15^\circ) R \rho C}{4\lambda} \right)} \right]^{-1}$$

matters arising

Fossil hominid femora

McHENRY and Corruccini used a canonical variates analysis to assess the affinities of five early hominid femora¹, two of which are complete (ER 1472, 1481, although the head of the former is damaged). The fossil hominids appeared to separate from living *Homo* on the fourth variate axis, accounting for only 4.7% of the variation. Nonetheless, the authors conclude that "there were at least two distinctive forms of hominids" represented. The authors state that head size is "heavily weighed" on this axis and suggest "large femoral heads might be the result of a widened birth canal." Yet, differences in femoral head size must be related to bone length to be meaningful and unambiguous; larger specimens will probably have larger heads.

Have they truly determined a relative head size variable? They argue that the femoral dimensions are adjusted using average within-taxon allometry coefficients "between each measurement and a standard size variable". But which coefficients might be used to standardise the early hominid measurements is problematic, for these combine relative neck length and shaft thickness dimensions which are unknown for any other hominid taxon, except possibly *H. erectus*.

Because they divide head diameter by neck length or shaft thickness, there is confusion about whether the head is relatively small, or the neck and shaft are relatively large. This underlies the different estimates of bone length in fragmentary early hominid specimens. For instance, McHenry² rejected an estimate of early hominid femoral length based on a regression from head size³ because, he argued, the heads are relatively small. Yet, he used a multiple regression to predict bone length giving much longer determinations based in part on neck length², although later realising that the necks are relatively long¹. Thus, the necessity of relying on those few specimens with known or reconstructable shaft lengths is underscored: shaft length is the only size measure which does not

require choice between neck or other shaft measures to estimate size in an analysis intended to determine whether or not the heads were relatively small. Unfortunately, only one published specimen requires no reconstruction to avoid this circularity (ER

Libben mean. The only other specimen that can be considered is STS 14, a very damaged proximal femur with a reconstructed length of 285 mm (ref. 5). (The estimate used here adds a portion of the head diameter inadvertently left off the published length of

Table 1 Biomechanical neck length and head diameter relative to femur length in a sample of 50 Libben Amerinds

	Relative biomechanical neck length	Relative head diameter
Mean $\pm$ (s.d.)	0.154 (0.008)	0.100 (0.005)
Maximum	0.177	0.114
Minimum	0.135	0.091

These data were provided by C. O. Lovejoy.

1481), although three others make possible additional estimations.

When 50 males and females from the Libben Amerind collection (Table 1) are used for comparison, relative biomechanical⁴ neck length for ER 1481 is 3.25 standard deviations above the Libben mean, and relative head diameter 1.80 standard deviations above the mean (Table 2). The next best specimen is ER 1472. Length is known but damage to the head prevents accurate head or biomechanical neck length measurements. As preserved, relative neck length is too short, but is still 1.00 standard deviation above

276mm). The neck is broken just before the junction with the head and has been repaired, although not reconstructed with plaster. The head diameter is reconstructed, but constrained because it must fit the associated acetabulum. Relative neck length is 3.25 standard deviations above the Libben mean, and relative head size 1.80 standard deviations. No attempt has been made to reconstruct femur length in other specimens with heads, since the various shaft diameters show a very low correlation with length in specimens with known or reconstructable lengths.

Table 2 Relative biomechanical neck length and head diameter in early fossil hominids compared with Libben

	Relative biomechanical neck length		Relative head diameter	
	Calculated	Difference from Libben mean in s.d.	Calculated	Difference from Libben mean in s.d.
ER 1481	0.180	+3.25	0.109	+1.80
ER 1472	> 0.162	> +1.00		
STS 14	0.182	+3.50	0.109	+1.80
ER 999	0.164	+1.25		

These measurements were taken by me.

Libben mean. ER 999, from above the Chari tuff, (1.34 Mys) is probably *H. erectus*; the relative neck length is 1.25 standard deviations above the

In sum, for all the known or reasonably reconstructable specimens, neck length is relatively very large compared with living humans and

Table 3 Comparison of innominate measurements normalised to acetabulum height

	Libben mean	σ	Distance from Libben mean in s.d.			
			STS 14	SK 3155	SK 50	OH 28
Ratio to acetabulum height of:						
Ilium height	2.33	0.14	+2.8	> +3.4*		
Greater sciatic to anterior notch	1.37	0.08	-2.3	-0.05	+0.46	-1.52
Anterior inferior to ischial spine	1.98	0.09	-1.3	-2.2		-2.9
Anterior inferior to posterior superior spine	2.57	0.14	-2.4	1.7		-0.4
Functional length of ischium	1.45	0.08	-3.8	-4.9	-0.8	-4.5

These measurements were taken by me. The functional length of the ischium⁴ is measured on both the Australopithecines and the Libben sample; the breaks on the STS 14 ischium and on the SK 3155 ischium minimise the measurement. The Libben sample size is 30. That some of these measures which cross, or almost cross the acetabulum differ considerably from the Libben mean when normalised by acetabulum size further emphasises the differences in proportion; when normalised to the acetabulum the iliac blades are relatively large and most other measures small, but normalised to the ilia everything else is extremely small.

*Addition of the unfused iliac crest would make this larger.

relative head size is, if anything, above the human mean⁵. These head, neck and shaft length relations are shown by two femora attributed to "*Homo*," and one attributed to "*Australopithecus*." Moreover, judging from the published photographs they also characterise the complete Afar hominid femur attributed to "*Australopithecus*"⁶. Former claims of relatively small head size come from comparison of head diameter with shaft diameter in incomplete specimens. Analysis of femora of known or reconstructable lengths shows that the shaft diameters were relatively large.

The demonstration that the heads were not relatively small calls to question the claim that the acetabula were relatively small¹, since of all pelvic dimensions the acetabulum has the highest correlation with femur length (0.840 for 30 Libben Amerinds). Schultz⁷ demonstrated the large relative size of the STS 14 and reconstructed SK 50 acetabula. When various comparable innominate measurements are normalised to acetabulum height (Table 3), and compared with the Libben sample, only relative ilium height is consistently above the Libben mean, as expected given the greater amount of iliac flare in the early hominids⁴. Iliac breadths are generally below the Libben mean, except for one measure of SK 3155, and contrary to "conventional knowledge", the functional length of the ischium is relatively short. The *H. erectus* innominate follows the australopithecine pattern. Only if normalised to iliac height would the early hominid acetabulum appear relatively small, and the other measurements would be relatively yet further below the Libben mean. Given the above, it is far more likely that australopithecine acetabula are human-like relative to body size, and the ilia are relatively long.

In sum, the distinguishing features of the early hominid hip complex reveal a pattern of form differing from living humans because of a combination of narrower birth canal and

markedly greater muscular activity, but not differing in locomotor capacity.

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McHENRY AND CORRUCINI REPLY—The discussion here¹ is about different fossils. We show that taken together the relative proportions of the proximal ends of KNM-ER 1481 and 1472 are distinguishable from those of SK 82 and 97 and KNM-ER 1504. Wolpoff argues¹ that the reconstructed femoral head diameter and neck length relative to reconstructed femoral length in Sts 14 are similar to KNM-ER 1481. We feel that a meaningful and unambiguous argument should not be based upon measurements of a fossil (Sts 14 femur) described as "a useless jumble of glued fragments surmounted by a crude plaster head and neck"². Nor should the length of a half missing ischium (SK 3155)³⁻⁶ be used to bolster such an argument.

A few points need to be clarified. (1) Our analysis is based on both univariate and canonical variates analysis and the results agree. (2) The variation on the fourth canonical variate is not interpreted by us as the result of femoral head size only. In fact, greater trochanter projection has the highest correlation with that variate, followed by neck length and femoral head size. The canonical variate is complex, reflecting the complexity of interrelationships among the variables. (3) Because variance scales to the number of subjects, one would not expect a high percentage of

variance on the fourth canonical variate. The fourth variate contributes substantially to the multivariate distances between subjects. (4) KNM-ER 1481 does indeed have a relatively big head, which is just our point because it represents the *Homo* taxon. The ratio of femoral head size to length in KNM-ER 1472 is 0.10 (ref. 7), which is exactly the Libben mean. This specimen is also classified as *Homo*. The only specimen classified as *Australopithecus* to which Wolpoff refers in regards femoral head size and length is Sts 14, but this specimen is missing both its head and its distal end⁴. (5) Walker⁵ did not conclude that the "relative head size . . . is above the human mean". He showed that his composite and reconstructed australopithecine femur has a ratio of head size to length slightly below the human mean.

In our view canonical variates analysis is one more useful tool for analysing complex biological shape. The method allows one to take into account variability within taxa, it reduces the complexity of interrelated shapes and sizes to understandable dimensions, and it allows an anatomical region to be treated as a total complex (inasmuch as the available fossil material will allow).

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Agglutinins for complement-coated sheep erythrocytes

SALIVARY 'IgA Immunoconglutinins', which reportedly bind both altered and native C3, have generated considerable interest^{1,2}. Unfortunately, the methods so far described for their purification are somewhat cumbersome, as they involve incubation of complement-coated sensitised sheep cells with saliva and subsequent elution of the 'IgA immunoconglutinins' with a chelating buffer. In our laboratory we have used a more classical approach, first fractionating parotid saliva on BioGel P100, then subfractionating each peak by DEAE-cellulose chromatography and testing the fractions for their reactions with complement and complement-coated cells (Fig. 1). (The details of the purification of salivary complement-reactive factors will be published elsewhere³.)

Our procedure has obvious advantages over that of Price *et al.*¹. Since Ca²⁺-dependent IgA agglutinins for sheep cells are present in low titre in salivary secretions^{3,4}, it is possible that those authors isolated secretory IgA attached not only to the human C3 on

erythrocytes would tend to confuse qualitative and quantitative agglutination results. Furthermore, preliminary evidence suggests that salivary C3

Matters arising

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and C4 agglutinins can be separated by DEAE subfractionation of the BioGel P100 peaks, with some salivary fractions demonstrating agglutinating activity for complement-coated sensitised erythrocytes and yet containing no detectable α -chain antigens.

Using purified saliva fractions we have found that saliva contains agglu-

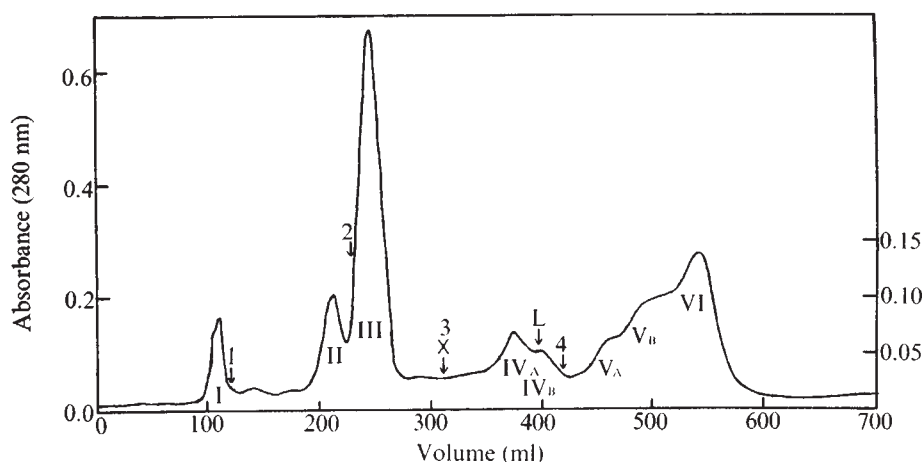


Fig. 1 Molecular sieve elution pattern of pooled parotid saliva chromatographed on BioGel P100. The elution buffer was 0.1 M Tris-0.2 M NaCl, pH 8.1, with a flow rate of approximately 6 ml h⁻¹. Column bed dimensions were 2.5 × 96.5 cm with a volume of 475 ml. The saliva was dialysed against the column buffer using 3,000 m.w. cutoff tubing (Thomas) before concentrating (Amicon apparatus, UMO5). Five millilitres of sample was added to the column. Salivary fractions (SFP) are labelled with Roman numerals. Numbered arrows refer to marker protein elution volumes; the proteins used were bovine albumin, chymotrypsinogen A, insulin, and bovine trypsin inhibitor, for arrows 1-4 respectively. The arrow labelled L represents the elution volume of human lysozyme, which was verified independently, and X denotes the scale change position.

the sensitised erythrocytes but also to the erythrocytes themselves. Contamination with IgA antibodies and non-specific agglutinins for sensitised

tinins for IgG- and IgM-coated erythrocytes, located in SFPIV and SFP II respectively (Fig. 1). α -chain antigens were found only in SFPI.

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PRICE *et al.* REPLY.—The claim by Boackle *et al.*¹ that their purification procedure "has obvious advantages over that of Price *et al.*" is based on the erroneous assumption that calcium-dependent IgA agglutinins to sheep erythrocytes are present in high titre in salivary secretions. In support of this statement they refer to the work of Tönder and Larsen². The IgA agglutinins described by these authors, however, were not calcium dependent and were directed against rabbit rather than sheep erythrocytes. Agglutinins to sheep erythrocytes were looked for and found in only four of 17 samples of saliva, the highest titre being 1:4. Other workers have shown that agglutinins to sensitised sheep erythrocytes are present in low titre in adult saliva, but these agglutinins are not calcium dependent^{3,4}.

Contamination with IgA antibodies directed against the sensitised erythrocytes would therefore not occur when EDTA was used to elute the anti-C3 antibodies from the cell surface. A simple way of preventing possible contamination by calcium-dependent agglutinins would be to incubate the saliva with sensitised erythrocytes before incubation with complement-coated cells.

Other workers who may be interested in studying these antibodies will decide whether EDTA elution from washed complement-coated erythrocytes (a procedure which may be performed in 2-3 h) or fractionating on BioGel P100 and then subfractionating on DEAE-cellulose is the less cumbersome method of purification.

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reviews

Geological survey of Greenland

J. Sutton

Geology of Greenland. Edited by Escher and Watt. Pp. 603. (Geological Survey of Greenland: Østervoldgade 10, DK-1350 Copenhagen K, Denmark, 1976.) Dkr.195 inc. postage.

COMPARED with many parts of the Solar System, the Earth is a protean creature, in a constant state of change. With such activity and destruction, Earth-bound geologists can never hope to find a landscape carpeted, as on the Moon, with fragments which have lain undisturbed for 3,000 Myr. Yet just occasionally geological circumstances combine to provide an unusually clear view of the geological past. The coastlines of Greenland, scraped bare by an ice sheet which has now drawn back to reveal fresh rock, provide unparalleled opportunities for geological research. This splendid book shows how successfully the Geological Survey of Greenland has grasped these opportunities during its first 30 years of activity.

As Dr Ellitsgaard-Rasmussen, who has directed the Survey for most of this time, points out, the Arctic climate and isolated situation require the mounting of special expeditions to carry out geological research in Greenland. The twenty-one chapters provided by some thirty contributors, most of whom have worked with the Survey, show how ably the staff have mastered logistical problems. With no fuss and great efficiency the Survey moves, feeds and supports its geological parties who are thus able to concentrate on scientific work, and who have surveyed, at least in outline, the entire mountainous seaboard bordering the world's largest island.

The opening sentence of the Director's preface puts the matter with characteristic plainness and brevity: "*Geology of Greenland*", he writes, "aims to provide a concise modern account of nearly all aspects of Greenland's geology". It does precisely this, drawing almost entirely on recent work while recognising the achievements of the pioneers, from the days of Giesecke in the early nineteenth century to the expeditions of Lauge Koch, Wegmann, Watkins and Wager more than a hundred years later.

Every chapter comes at first hand from experts who have been repeatedly to the terrain they describe. Each provides a readable narrative between a dozen and sixty pages in length, excellently illustrated. The figures (there are 470) include a variety of geological sketch maps and photographs that demonstrate the clarity with which geological phenomena are displayed in Greenland. The authors reflect the international nature of the enterprise. The Survey collaborates with more than fifty Universities and Institutes, and moreover does so with a generosity and openness which has attracted able scientists from many countries. The heart of the undertaking lies, however, in Denmark. It is Danish resources and leadership that have brought the Geological Survey of Greenland to its present eminence. Readers of this book have plenty of material on which to assess this judgement.

The opening chapters will appeal to the student of crystalline rocks. Here are described the Archaean and Proterozoic rocks which form the mass of Greenland. The main divisions established in Greenland can be extended into Canada and north-western Europe, and so provide a key to a unified view of the Precambrian of much of the Northern Hemisphere. Of even greater importance, perhaps, is the insight into geological processes of those times, made possible by careful study of excellently displayed terrain. These chapters could influence scientific thought in the way the British Geological Survey's work on Tertiary volcanic centres early this century influenced

the development of petrology. In each instance accounts of newly discovered phenomena prompt inquiries into their origin. One thinks of Bowen's use of British investigations some fifty years ago. An equal opportunity is provided here.

But this is no more than the first course. There follow chapters on the younger rocks of the east, north and west coasts, each of which developed somewhat differently, although all illustrate what may happen before and during the separation of continental blocks. The evolution of eastern Greenland is taken in three excellent chapters from late Precambrian times to the late Tertiary. A similar time span is covered in an account of northern Greenland which provides the first synthesis of that region based on detailed observation. Petroleum prospects, although still at an early stage, are suggested by the accounts of sedimentation around the Greenland coasts, which include short reference to commercial work offshore.

An account of the glaciation and Quaternary history of Greenland completes the geological record of this remarkable island. About fifty pages are devoted to economic minerals, coal and petroleum, followed by an equal number in which plant and vertebrate fossils are described. The book ends with an account of the kimberlites of western Greenland.

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Essence of Iceland

The Landscapes of Iceland: Types and Regions. By H. Preusser. Pp.xvi+363. (Junk: The Hague, The Netherlands, 1976.) DGu120.00.

REGIONAL geography encompasses the systematic patterns of landscape: the physical form and structure, the heat and water available through the seasons and the resulting biotic and human development. Most essays in regional

geography describe the physical characteristics of landscape as a platform or stage on which the life styles of work and play are integrated into a cultural pattern over the environment, increasingly controlled by complex economic forces. If these components are well drawn and emphasis is duly apportioned to each, and differences across regional boundaries are correctly discerned, the essay is then greater than the sum of

its parts and the story told gives the essence, the *entité*, of the region.

This book describes the landscape in great detail and is thorough in defining type areas and the effect of man. The cultural landscape is given less attention because the low population density compels less human emphasis. The book is a doctoral thesis based on an intensive search of the literature (there are 30 pages of bibliography), a detailed interpretation of maps, diagrams, air and satellite photographs and three extensive visits. The author has been fascinated by the sudden changes that the traveller sees in this land of ice and fire, with its awesome interior plateau more like a moonscape than any landscape on Earth.

The first part (100 pages) of the book is a national compendium, defining terms, describing landscape features, mapping climatic elements and listing population and employment changes

and their products up to 1970. The second part of the book (40 pages) describes nine main landscape types. The third part (about half the book) extends this, with 30 geographically located regions, some with further subdivision. It is not easy to know where the author is in these descriptions, although the many diagrams and sketch maps are a great help. It is therefore essential to have the nine Icelandic maps (1:250,000) alongside the book. To capture the spirit of place, the reader should also have some of the many beautifully produced volumes of colour photographs of Iceland.

The book is a scholarly work and is, considering how crammed with information and references, remarkably easy to read.

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State of the oceans

The Health of the Oceans. By Edward D. Goldberg. Pp. 172. (Unesco Press: Paris, 1976.)

EDWARD GOLDBERG'S book arose as a result of a recommendation made in 1973 at the time of the first meeting of the Intergovernmental Oceanographic Commission's International Co-ordination Group for the Global Investigation of Pollution in the Marine Environment. As originally conceived, it was intended to fulfil the role of a baseline, or statement on the status of pollution in the marine environment, against which to judge the changing health of the oceans within a continuing review of marine pollution as part of the Global Investigation of Pollution in the Marine Environment. A great deal of useful information to that end is contained within the ten chapters, but the book has nevertheless been written against a much broader conception of the problem than was originally intended.

There is an effort to produce a health status report; but also much of a speculative nature written to examine causes, to reflect on philosophy and to make tentative suggestions about future problems and their solutions. The book dwells at length on the lessons to be learned from the successful regulation of radioactive waste disposal to the oceans, on the mercury problem, and on DDT and oil. The author clearly believes that much could be gained by attacking other pollution problems along similar lines to those used in the UK in relation to radio-

activity, including the application of 'critical path' philosophy to the design and implementation of monitoring programmes; a suggestion with which the reviewer has much sympathy.

Halogenated hydrocarbons, radioactivity, heavy metals and oil all receive treatment in separate chapters, supported by chapters which deal collectively with marine pollution dynamics, modelling (especially mass balance studies), monitoring and prediction. Among the problems which obviously concern the author, and which he returns to throughout the text, are the long term significance of small changes in pollutant concentration in the oceans, the difficulty in unequivocally establishing such trends, and our reaction time in taking remedial action when the system's reaction time itself may be long and, as a consequence, re-establishment of normal concentrations may take a long time: a time during which damage to the system may appear which will take longer to rectify and which may have serious consequences as yet unpredictable.

There is a carefully written preface by Alan Holden, and readers of the book would do well to peruse it before tackling the text. One must conclude, however, that Professor Goldberg has picked his way through something of a minefield without much more than superficial wounds, and produced a thought-provoking text on the way.

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Updating nematodes

Physiology of Nematodes. Second Edition. By D. L. Lee and H. J. Atkinson. Pp. x+215. (Macmillan: London, October, 1976.) £5.95.

WHEN Donald L. Lee wrote the first edition of this book it was a milestone. Although of modest size, it was the first attempt to organise the subject, stimulated great interest and highlighted critical areas for the research efforts of others. Now, with his colleague, Howard J. Atkinson, a second edition has been published. In the eleven years between the editions and partly because of the impact of the first, there have been numerous papers, reviews and books on nematode physiology. This new edition still presents an excellent introduction for undergraduates, as it is designed to do, but those professionally involved with nematodes will spot some over-generalisations and omissions.

The chapters include: introduction; cuticle, moulting and growth; feeding and digestive physiology; metabolism (a particularly comprehensive section); osmotic and ionic regulation; excretion; reproductive physiology and hatching; neuromuscular physiology; locomotion; sense organs and behaviour. There are 77 figures, less than 40 of which were in the first edition, and there are 246 references instead of the earlier 161. The references are only representative and no attempt is made to credit individual workers in particular fields. There are few typographical errors but nematode names are occasionally mis-spelt (four in chapter 4) and there are seven errors in the appendix listing nematodes. The text and figures have the same clear, concise and very readable features of the first edition.

Specific points are sometimes unclear: nematodes are still credited with constant cell numbers but this is a much compromised notion now (p27). Predatory nematodes are poorly discussed in the light of the most recent studies (p44). Various reports have shown that the 'lens' of ocelli is, in fact, the photoreceptive rhabdomere and that no lens exists. The figure of *Deontostoma californicum* with a lens is particularly unfortunate as this was the species first shown not to have a 'lens'. Photic response for two species is ignored (p68) which makes incorrect the statements on their behaviour.

At £5.95 this book deserves to enjoy good sales, and it will be widely read.

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Liquid and solid helium

The Physics of Liquid and Solid Helium. Part 1. (Interscience Monographs and Texts in Physics and Astronomy, Vol. 29.) by K. H. Bennemann and J. B. Ketterson, Pp. 589. (Wiley-Interscience: New York and London, June 1976.) \$35.35; £21.

SOME books are to be read as a whole; others may to greater advantage be dipped into with discretion. This book, consisting of six chapters each of which amounts to a relatively self-contained review article on a specialised topic, clearly belongs to the latter class.

The first chapter, by Khalatnikov on the phenomenological theory of superfluid ^4He has much in common with his frequently cited monograph *An Introduction to the Theory of Superfluidity* (Benjamin, New York, 1965). There follow five detailed and carefully constructed reviews: by Ahlers on experiments near the superfluid transition; by Fetter on ions and vortices; by Stephen on light scattering; by Woo on microscopic calculations; and by Varma and Werthamer on solid helium. Each of these chapters has its own bibliography which, in most cases, is extensive enough to be really useful to anybody working in or wishing to enter the field in question. Fetter, for example, includes more than 500 separate citations.

It is not a book for beginners. Khalatnikov's chapter, although admirable in itself, is too succinct, and too limited in scope, to constitute an adequate introduction to the material which follows. Graduate students will therefore be well advised to read an introductory text, such as the excellent little book by Tilley and Tilley (*Superfluidity and Superconductivity*, van Nostrand Reinhold, London, 1974), before embarking on the sections of the present volume relevant to their research.

Apart from the first, all the topics covered are ones in which there has been considerable progress in recent years. These substantial review articles will thus be of value to research workers in assessing the current position and deciding where further investigations are still required. The absence of a chapter dealing with the rapidly developing field of superfluid ^3He is naturally disappointing, but a certain loss of topicality and impact was inevitable in view of the four years' delay between completion of writing and publication. In most cases the authors have done what they can at the proof stage, through incorporation of

addenda and additional references, to update their original material to 1974 or so. Although the book is clearly a must for libraries, the extent of individual sales is likely, on account of the price, to be somewhat limited. This is rather a pity, because there will undoubtedly be many who would appreciate personal copies of particular chapters, but who will be disinclined, or unable, to pay for all the others which may be of less interest to them.

Part II which, it is promised, will include a couple of chapters on superfluid ^3He will be awaited with eager anticipation. **P. V. E. McClintock**

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Flavour of gene expression

Control of Gene Expression. By Norman Maclean. Pp. xi+348. (Academic: London and New York, May 1976.) £7.80; \$19.75.

POSSIBLY because this book arrived on my desk around holiday time I viewed it as a sort of tourist menu attempting to offer a wide array of characteristic foods as an introduction to the culinary arts of a new country. The broad title is perhaps a little misleading for the bulk of the book presents a series of concise introductions to the numerous and diverse systems which have been used to study particular aspects of gene expression in eukaryotes. These systems are catalogued under three headings: systems involving control of a single type of protein; systems described as being of limited complexity; and systems classified as "not well understood in molecular terms". These three chapters constitute the meat of the book, the merits of which must rest on the strength of these three chapters.

In describing the systems involving one type of protein the author has clearly set himself a difficult task. The result is an acceptable précis, brief enough to be read by the enthusiastic undergraduate; barely detailed enough for that undergraduate's teacher, but dotted with key references for further reading. Moreover the author has commented in each case on some of the uses to which each system has been, or might be, put.

There is obviously a continuum between systems of limited complexity and systems described as "not well understood in molecular terms". The point at which the author draws the line between these two categories must be arbitrary. It is in these two chapters

that I think the book will come into its own as an introductory text for biochemistry and molecular biology undergraduates. The wide range of fascinating systems are dealt with briefly but interestingly. If any of these accounts serves to lead students trained in the molecular sciences into the study of one of these fascinating systems then the author will be able to congratulate himself.

The book leads off with a chapter on control of gene expression and its level of action, followed by a brief account of gene expression in prokaryotes. At the end there are chapters on RNA involvement in gene expression and general concepts of gene regulation. Each of these chapters is well laid out and might be taken as a separate reading, although read together some parts are a little repetitious.

As a tourist menu of the area of gene expression in eukaryotes the book succeeds in giving a taste of many systems, some fashionable and some less so, and in a number of instances it manages to convey authentic flavour.

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Environmental Chemistry. By John W. Moore and Elizabeth A. Moore. Pp. xv+500. (Academic: New York and London, April 1976.) \$27.50; £15.10.

MANY environmental problems have been initiated by the failure of decision-makers to consider problems in their entirety and the failure of specialists to make relevant information both available and understandable. American science students having pursued the popular, mind-broadening course on which this book is based will be better able to fulfil their roles in both science and society irrespective of the careers they choose. Most suited to the interests of chemists having acquired a basic knowledge of kinetics and thermodynamics, the book should be considered essential reading for degree level courses in chemistry or environmental science. Physicochemical principles are applied to a breadth of practical non-ideal systems with economic, historical, and social commentary in an enjoyable and readable manner.

Following an introductory section on chemical evolution, the main text is based squarely on the alchemical elementary concepts of fire (energy), air, earth and water illustrated using modern issues. Nuclear reactor safety, stratospheric ozone depletion, phosphate eutrophication and use of pesticides are some of the controversial subjects discussed in a balanced way from an American viewpoint. Topicality will date the book rapidly, however, as evidenced by the now rejected Ca-Hg-Br system quoted to explain the hydrogen economy. Exercises and further reading suggestions are included in each chapter. The author's style, free from American phraseology, will appeal to students but many will consider the Moores' first textbook rather expensive.

I. R. McKinley

Examination and Analysis of Starch and Starch Products. Edited by J. A. Radley. p. vii+220. (Applied Science: London, 1976.) £15.00.

THIS is a practical book giving a description of various starches and the methods for identifying and characterising them.

G. E. Moss describes methods for examining starches in the light microscope. The appearance of various starches is given with numerous illustrative photographs. A table provides information on the microscopic appearance of 32 different starches. D. J. Gallant and C. Sterling describe

both the transmission and scanning electron microscopes and their application to starches. A. H. deVilligen discusses the rheology of different starches. A chapter by the editor follows, in which rheological measurements are discussed along with a description of various commercial viscometers. Procedures are also discussed for measuring gels, colour and several other properties. F. A. Lyne describes methods for determining moisture, mineral matter, fats, protein, alkali number, amylose content carboxyl groups and so on and also provides procedures for the

Books brief

determination of starch in various products. J. van der Bij provides methods for the analyses of starch ethers and esters.

Each chapter has many references to the fundamental literature. The book should be useful in analytical laboratories concerned with starch analysis. Although detailed methods are not often given, sufficient references are present to lead to the appropriate details where needed.

Roy L. Whistler

The World Computer Chess Championship. Stockholm, 1974. By Jean E. Hayes and David N. L. Levy. Pp. v+105. (Edinburgh University: Edinburgh, May 1976.) £3.75.

THE first World Computer Chess Championship took place in Stockholm in August 1974, and resulted in a closely contested victory for the Soviet Union's 'Kaissa' program, ahead of several American rivals. This book, written by a professional chess master and a research worker in artificial intelligence, combines a report of the tournament games with a brief introduction to the problems of programming a complex human skill into a computer.

Although the account presents the intended material in a well organised manner, I felt that it has fallen short of what might have been achieved. The account of the games is rather pedestrian, following too closely the style of annotation appropriate to human chess, and laying insufficient

emphasis on the peculiar motives and aberrations of computer play. Furthermore, the delay of nearly two years between the tournament and the appearance of the book is far too long in such a rapidly developing subject, and this, coupled with the use of the obsolescent English Descriptive notation (which has all but disappeared from international chess) produces the impression of a historical document rather than an up-to-date report.

Compared with the much earlier report on the same tournament by Monroe Newborn, I find the present book difficult to recommend. It does however, have the merit of being considerably cheaper than the earlier report.

Nigel J. Holloway

Flora Europaea. Vol. 4: Plantaginaceae to Compositae (and Rubiaceae). By T. G. Tutin *et al.* Pp. xxix+505+5 maps. (Cambridge University: Cambridge: London and New York, August 1976.) £25.

THE production of *Flora Europaea* is a remarkable project in a number of respects. The geographical area and habitat diversity of the European continent makes it rich in plant species, so the description of its flora is a formidable task. It calls for a highly concise but informative text and in this respect *Flora Europaea* is eminently successful. It is also a credit to authors, editors and publishers in that the appearance of new volumes is a regular and frequent event.

Volume four is now in the bookshops and it maintains the standards of brevity set by its predecessors. The bulk of this volume is taken up with the family Compositae, which brings with it the taxonomic challenge of such genera as *Taraxacum* and *Hieracium* with their multitude of microspecies. The authors deal with these groups in a pleasantly conservative manner. Those of us who are consumers rather than producers of things taxonomic will welcome this agglomerative rather than divisive approach. There is one point, however, where a little splitting might have been welcome, that is in the case of *Hieracium pilosella*. There is a case, one would have thought, for its removal from this large genus.

As a source of concise information, relevant to the needs of taxonomists, ecologists and biogeographers this series is exemplary. The price is not unreasonable by modern standards, although subscribers may feel trapped in the general price escalation of the series.

Peter D. Moore

obituary

Lars Onsager, who died on October 5, 1976, was undoubtedly one of the great scientists of the 20th century. His publications were neither lengthy nor numerous. When he was awarded the Nobel prize in Chemistry for 1968 for his discovery of "Reciprocal relations in irreversible processes", the citation noted that the publication (two papers in *Physical Review*¹ containing 22 and 15 pages respectively) was one of the smallest ever to be awarded a Nobel prize.

Onsager's total publication list runs to some 60 papers, a number which can readily be matched by most university professors of science. But the quality of his publications is superb, and he did not put his name to anything trivial or unfinished. Besides non-equilibrium thermodynamics, a field initiated by his Nobel prize work, there are at least five fields his contributions to which ensure him a place of distinction. In the area of phase transitions many regard his exact solution of the two-dimensional Ising model as of comparable significance to his Nobel prize work; and in the theory of electrolytes, liquid helium, metals and ice, many of his ideas have by now been incorporated into standard texts. He also devoted thought and attention to the problem of turbulence in fluids. He provided a magnificent counter-example to the "publish or perish" philosophy which has influenced so many scientists during the past few decades; and there was a general consensus of opinion among those who had direct contact with him that the unpublished contents of his drawers would have sufficed to establish the reputations of quite a few scientists with less exacting standards than his own.

Despite the clarity of his thinking and writing Onsager was a poor lecturer, and usually pitched his exposition far above the heads of the audience. This was not due to any conscious desire to show off (which was quite alien to his nature), but to a complete lack of appreciation of the gap between himself and his listeners. An incident which occurred during his sabbatical leave at Cambridge in 1951–1952 illustrates this well. Onsager was asked to talk to the Kapitza Club about his work with Bruria Kaufman on the Ising model. The Kapitza Club (named after its founder) aims to provide a meeting-ground between theoretical and experimental physicists. In



Photograph taken by Z. R. Hasan, a graduate student of Temple University, during a visit by Onsager a few months before his death.

his briefing Onsager was warned that since there would be experimentalists present he should phrase his talk in a language which would be intelligible to them. Nevertheless, he started with the mathematics of spinors. After a few minutes the thought occurred to him that some of the audience might not have come across spinors before, and he turned to ask if there was anyone present who did not know what a spinor was. One or two brave spirits raised their hands, and Onsager said "Right, I will tell you; spinors are matrices isomorphic with the rotation group in three dimensions" and he continued his talk assuming that this was a sufficient explanation for an experimental physicist!

During the same year he gave a seminar at Oxford about his ideas on the theory of liquid helium, and on this occasion even the theorists were at a loss in trying to follow his arguments. Onsager's final comment in reply to a discussion question was "The results are not bad when you consider the enormity of the swindle which I have perpetrated". It was only a few years later when Feynman's theory of liquid helium appeared with acknowledgements to Onsager's ideas that light was shed retrospectively on what Onsager had been trying to convey in the seminar.

In spontaneous discussion at conferences he came over better, and was

often entertaining as well as informative. Onsager would get up at the end of a long and sophisticated lecture and say "I think Dr X has tackled this problem in a very effective manner, and his first approximation is in the right direction; if he proceeds to a second approximation he should find the following . . ." And he had an endearing habit of going to the front of a distinguished audience, and turning to them with a beaming smile to ask practical down-to-earth questions like a schoolteacher. "Does anyone know what is the best substance to use to illustrate A, B, C? Well, it is sulphur, because . . ."

His knowledge of different aspects of science was immense. When taken round a laboratory he would comment pertinently on every detail of the experimental set-up, to the great surprise of the experimentalists who knew of him only as an abstruse theoretician. And he took it for granted that every research scientist would be familiar with all text-book material—"But it's in all the standard books on palaeontology".

In private discussion it was much easier to communicate with Onsager provided that you were courageous enough to persist in questioning when you did not understand. He would drop the level one stage at a time until the gap could be bridged. Thus he had many disciples and collaborators who uniformly testify to his generosity, and to the inspiration of working with him on scientific problems.

Onsager was born in Norway in 1903. He entered the Norges Tekniske Høgskole in Trondheim in 1920; his first research ideas developed here and were concerned with the theory of electrolytes. After graduating in 1925 he went to the Eidgenössische Technische Hochschule in Zurich to work with Debye and Hückel, and his first publications² were devoted to a revision of the Debye–Hückel theory.

In fact, it was the combination of diffusion and electrical conduction in electrolytes for which he found that reciprocal relations were satisfied which led Onsager to a deeper study of their origin. Although the second law of thermodynamics gives precise information when applied to equilibrium phenomena and reversible processes, for irreversible processes it provides only the qualitative information that the entropy increases. Kelvin had attempted to extend thermodynamics

beyond the equilibrium state in his theory of thermoelectricity, and Helmholtz had derived a "Principle of least dissipation" which Onsager recognised as being equivalent to the reciprocal relations. Onsager had also been thinking about the condition of detailed balancing used widely in the theory of chemical reactions and usually taken to be a consequence of the second law. He became convinced that there was nothing in the second law to prevent equilibrium being maintained by cyclic processes when there were more than two independent reactions. A new hypothesis of microscopic reversibility was needed to exclude this possibility.

In his Nobel prize papers¹ published in 1931 Onsager tied all these ideas together, and showed that the reciprocal relations and principle of least dissipation could be derived from this hypothesis. He thereby established a new branch of thermodynamics. For some years little attention was paid to his papers; but in the post-war era the subject of irreversible thermodynamics gained momentum steadily and numerous applications arose in physics, chemistry and biology as well as in technology.

In 1928 Onsager had emigrated to the USA. He spent one term at Johns Hopkins University and five years at Brown University. In 1933 he went to Yale where he stayed until his retirement in 1972; from 1945–72 he served as Josiah Willard Gibbs Professor of Theoretical Chemistry.

His classic paper giving the solution of the two-dimensional Ising model² was published in 1944. The paper was a mathematical *tour de force* and amongst other skills showed remarkable dexterity in handling elliptic and elliptic modular functions. (Onsager once confided to Elliott Montroll that whilst a student he had worked through every example in Whittaker and Watson's *Modern Analysis*!) For the first time the exact statistical mechanics of a realistic model of interacting systems became available. Ideas about the nature of critical points and the behaviour of thermodynamic functions in their neighbourhood had to be completely revised; the publication laid the foundation of the modern era of phase transitions.

The 1944 paper dealt with thermodynamic functions in equilibrium. Of no less importance are the correlations between spins at different distances, and in collaboration with Bruria Kaufman³ Onsager published a paper in 1949 devoted to their behaviour. In the same year he announced cryptically at a conference in Florence⁴ that he and B. Kaufman had also solved the problem of long-range order for a rectangular net and that it was simply $(1-k^2)^{1/8}$ (where k is a simple function of the

interactions). This was a result of the greatest importance, which was re-derived independently by Yang⁵ in 1952. Onsager never published any details of his derivation, and gave no clue to the mathematics he had used. It was only twenty years later at a conference in Gstaad to celebrate the 25th anniversary of the publication of his 1944 paper that some information was forthcoming⁷. In computing the long-range order he had been led to a general consideration of Toeplitz matrices, but he did not know how "to fill out the holes in the mathematics, the epsilons and deltas", and by the time he had achieved this satisfactorily he found that "the mathematicians got there first".

There are many jewels to be found among Onsager's publications and those of others which incorporate his ideas; like the duality relation in two-dimensional Ising nets⁸, the proof of the existence of a Bose-Einstein condensation in interacting systems⁹ (with Penrose), and a novel method of looking at the de Haas van Alphen effect¹⁰. He returned several times to the theory of electrolytes where his researches started (particularly in collaboration with Fuoss).

After retiring from Yale in 1972 Onsager went to the University of Miami. He remained healthy and vigorous until his death; the photograph was taken only a few months before he died.

Onsager held honorary doctorates of several universities, including Cambridge and Oxford. Among his many awards were the Rumford Medal of the American Academy of Arts and Sciences, the Lorentz Medal of the Royal Netherlands Academy of Sciences, and the National Science Medal. He was elected a Foreign Member of the Royal Society in 1975. His colourful personality will be missed particularly at international gatherings.

C. Domb

¹ Onsager, L., *Phys. Rev.*, **37**, 405–26; **38**, 2265–79 (1931).

² Onsager, L., *Z. Phys.*, **27**, 388–92 (1926); **28**, 277–98 (1927).

³ Onsager, L., *Phys. Rev.*, **65**, 117–49 (1944).

⁴ Kaufman, B., and Onsager, L., *Phys. Rev.*, **76**, 1244–52 (1949).

⁵ Discussion remark in *Nuovo Cim*, Ser. IX, **6**, (Suppl.) 261 (1949).

⁶ Yang, C. N., *Phys. Rev.*, **85**, 808–16 (1952).

⁷ Onsager, L., in *Critical Phenomena in Alloys, Magnets and Superconductors*, edit., Mills, R. E., Ascher, E., and Jaffee, R. L., McGraw-Hill (1971).

⁸ Wannier, G. H., *Rev. mod. Phys.*, **17**, 50–60 (1945).

⁹ Penrose, O., and Onsager, L., *Phys. Rev.*, **104**, 576–84 (1956).

¹⁰ Onsager, L., *Phil. Mag.*, **7**, 1006–8 (1952).

Dr William Nordberg, internationally known space scientist and Director of Applications at NASA's Goddard Space Flight Centre, near Washington, D.C., died on October 3, after a two year illness, from cancer. Born in 1930, Dr

Nordberg was educated at the University of Graz in Austria, his native country. He emigrated to the U.S.A. in 1953 and after working for the U.S. Army Signal Corps as an atmospheric physicist, particularly on the International Geophysical Year rocket programme, he joined the newly formed NASA at the Goddard Space Flight Centre in 1959. His first post there was head of the physical measurement section, Meteorology Branch in the Satellite Applications Division. From there he progressively rose to higher management positions until his appointment as Director of Applications in 1974.

From the beginning of the U.S. satellite programme Dr Nordberg applied his enormous enthusiasm and powers of leadership to pioneering the development of remote sensors for observing the atmosphere from space vehicles. He was involved in the instrumentation of the early Tiros weather satellites and many of the infra-red radiometric instruments on the first Nimbus meteorological research satellite were developed and built under his direction. He was quick to realise the potential of remote sounding measurements, not only for research on the atmosphere, but also for the investigation of the properties of the earth's surface, and under his leadership microwave instrumentation for mapping the distribution of sea ice and other important surface features was flown. The success of the Landsat satellites, which have carried remote sensors for mapping a wide range of earth resources, owed not a little to Dr Nordberg's foresight and ability to organise and to interest people from a wide range of scientific disciplines. He successfully co-ordinated the activities of 300 principal investigators from 38 countries in the use of Landsat data.

Dr Nordberg received a number of awards including NASA's highest award, the Distinguished Service Medal. In September of this year, shortly before his death, he was elected to be a Fellow of the American Meteorological Society, a fitting tribute to his pioneering work in space meteorology.

Bill Nordberg, through his extensive travels, made friends in almost every country of the world. He will always be remembered for his enormous energy, his infectious enthusiasm for science and his tremendous zest for life which continued even right through his long and trying illness. By his tragically early death the international space research community has lost an outstanding scientist and many of us have lost a true and valued friend.

He leaves his wife, and his parents and brother in Austria.

J. T. Houghton